	L #	Hits	Search Text	DBs	Time Stamp
1	L1	165	ikk$2	USPAT; US-PGPUB	2002/07/17 08:34
2	L2	23927	kinase$1	USPAT; US-PGPUB	2002/07/17 08:34
3	L3	52	1 same 2	USPAT; US-PGPUB	2002/07/17 08:34
4	L4	32	1 same (human or murine or mouse)	USPAT; US-PGPUB	2002/07/17 08:35
5	L5	20	3 and 4	USPAT; US-PGPUB	2002/07/17 08:35

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TITLE: Mutations in IKK gamma

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ABSTRACT:

The present invention relates to compositions and methods involving IKK.gamma. mutants. In particular, the present invention provides methods and compositions, including transgenic animals, suitable for use in determining means to treat, control, and/or prevent incontinentia pigmenti (IP). The present invention also provides methods to detect the presence of mutations in the IKK.gamma. gene and protein.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. .sctn.119(e) of provisional patent U.S. Ser. No. 60/212,438, filed on Jun. 16, 2000, which is herein incorporated by reference in its entirety for all purposes.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0007] IKK.gamma., also known as the nuclear factor-.kappa.B (NF-.kappa.B) essential modulator or NEMO, is the essential regulatory subunit of the I-.kappa.B **kinase (IKK**) and is encoded by an X-linked gene in mice and humans. It is required for NF-.kappa.B activation and resistance to tumor necrosis

factor (TNF)-induced apoptosis. Female mice heterozygous for IKK.gamma./NEMO deficiency develop unique dermatopathy, characterized by keratinocyte hyperproliferation, skin inflammation, hyperkeratosis, and increased apoptosis. Although Ikk.gamma..sup.+/- females eventually recover, Ikk.gamma..sup.- males die in utero. These symptoms and inheritance pattern are very similar to those of IP, a **human** genodermatosis that is syntenic with the IKK.gamma./NEMO locus. Indeed, biopsies and cells from IP patients exhibit defective IKK.gamma./NEMO expression, but normal expression of **IKK** catalytic subunits. This unique self-limiting disease, the first to be genetically linked to the **IKK** signaling pathway, is dependent upon X-chromosome inactivation. Although an understanding of the mechanism(s) is not necessary in order to use the present invention, the results obtained during the development of the present invention indicate that IKK.gamma./NEMO-deficient cells trigger an inflammatory reaction that eventually leads to the death of these cells.

Brief Description of Drawings Paragraph - DRTX:

[0012] FIG. 1 shows various aspects involved in the generation of IKK.gamma./NEMO-deficient mice. In Panel A, maps of the **mouse** Ikk.gamma./NEMO locus, targeting vectors, and the targeted allele generated by homologous integration of vector 1 are provided. In this Figure, exons 1 and 2 are indicated by the solid boxes. The translation start site, selection markers, hybridization probe (bold line), PCR screening primers (P1 and P2), and restriction enzyme sites are shown (B, BamHI; Bg, Bg/II; 47III, Eco47III; S, SalI). Panel B provides the results of Western blot analysis of **IKK** subunits in whole embryonic stem (ES) cell extracts. In this Figure, lane 1 contains wild-type, lane 2 contains Ikk.gamma..sup.- from KO1, lane 3 contains Ikk.gamma..sup.- ES2 from KO1, lane 4 contains Ikk.gamma..sup.- ES3 from KO2, and lane 5 contains Ikk.gamma..sup.- ES4 from KO2; IKK.gamma.(N) and IKK.gamma.(C) refer to antibodies directed against the N- and C-terminal regions of IKK.gamma./NEMO, respectively. Panel C provides results of experiments in which **murine** embryonic fibroblasts (MEFs) derived from wild-type and Ikk.gamma..sup.- E12 embryos were treated with 10 ng/ml tumor necrosis factors (TNF.alpha.) or 20 ng/ml interleukin-1 (IL-1). At the indicated times, cells were lysed and **IKK** activity (KA) was measured by immune complex **kinase** assay using IKK.alpha. antibodies and glutathione-s-transferase fusion protein, GST-1.kappa.B.alpha. (1-54), as a substrate. The reaction products were separated by a 10% SDS-PAGE, transferred to a membrane, and autoradiographed. The membrane was reprobed (Blot: IKK.alpha.) with a monoclonal antibody against IKK.alpha. as a loading control. Panel D shows the NF-.kappa.B binding activity in 20 .mu.g of whole cell extracts prepared as described in Example 3. NF-1 probe binding was used to control the quality and quantity of the nuclear protein extract. Panel E illustrates TNF.alpha.-induced cytotoxicity for wild-type and Ikk.gamma. MEFs treated with **mouse** TNF.alpha. (50 ng/ml). At the indicated time points, cells were fixed, stained with DAPI, and mounted with a coverslip. Values shown are percentages of apoptotic nuclei scored using a fluorescent microscope.

Brief Description of Drawings Paragraph - DRTX:

[0018] FIG. 7 shows the expression of **IKK** subunits in **human** skin biopsies and fibroblasts. Panel A shows (400X) sections from normal individuals and IP patients, individuals with X-linked ichthyosis, and individuals with psoriasis. These sections were stained using specific antibodies against IKK.alpha., IKK.beta., or IKK.gamma./NEMO. The immune complexes were visualized by chromogen reaction, resulting in a brown precipitate. Sections were counterstained with hematoxylin. Panel B shows IKK.gamma./NEMO expression in **human** fibroblasts. In this Panel, lane 1 contains HEL229, lane 2 contains W138, lane 3 contains a fibroblast sample from IP patient 1, and lane 4 contains a fibroblast sample from IP patient 2.

Brief Description of Drawings Paragraph - DRTX:

[0024] FIG. 13 shows results obtained from a biochemical analysis of wild type and IP fibroblasts. Panel A shows that IP fibroblasts exhibit a reduction in cytokine-induced **IKK kinase** activty, while Panel B shows that IP fibroblasts exhibit a reduction in cytokine-induced NF-.kappa.B binding activity. As shown in Panel C, the reduction in **IKK kinase** and NF-.kappa.B binding activities were not due to a lack of p50 or p65 subunits.

Detail Description Paragraph - DETX:

[0049] In view of the marked similarity in symptoms, inheritance and chromosomal location between **human** IP and the Ikk.gamma./NEMO mutations in the **mouse,** experiments were conducted during the development of the present invention to examine the expression of **IKK** subunits in skin biopsies and fibroblasts from IP patients. While biopsies from normal individuals were found to be invariably and uniformly positive for expression of all **IKK** subunits, biopsies taken from affected areas of IP patients were positive for IKK.alpha. and IKK.beta., but expression of IKK.gamma./NEMO was strikingly reduced. Fibroblasts taken from a male fetus and a newborn, whose mother was diagnosed with IP, were found to be completely deficient in expression of IKK.gamma./NEMO. Thus, it is believed that **human** IP is caused by defective IKK.gamma./NEMO expression. In both **mouse and human** female carriers, pathogenesis is contemplated as being dependent upon sequential hyperproliferation and apoptosis of IKK.gamma./NEMO-deficient cells that are generated through X-chromosome inactivation (Lyonization). However, an understanding of the mechanism(s) is not necessary in order to make and use the present invention.

Detail Description Paragraph - DETX:

[0054] Unlike the genes coding for the catalytic subunits of the **IKK** complex, the IKK.gamma./NEMO gene, which codes for the essential IKK.gamma./NEMO regulatory subunit is located on the X-chromosome. Due to this chromosomal location and random X chromosome inactivation (Lyonization), loss-of-function Ikk.gamma./NEMO mutations result in different phenotypes in female and male mice. While mutant males die in utero, as expected for a complete **IKK** and NF-.kappa.B deficiency (Li et al., Proc. Natl. Acad. Sci. USA 96:1042-1047

[1999], Li et al., Genes Dev. 13:1322-1328 [1999]; and Tanaka et al. Immunity 10:421-429 [1999]), heterozygous mutant females are born alive, but display a severe, yet transient, multi-organ disease. The cutaneous signs of the disease detected in Ikk.gamma..sup.+/- female mice are very similar to those observed in **human** patients suffering from IP. Both Ikk.gamma..sup.+/- mice and female IP patients eventually recover, reach sexual maturity, and can transmit the disease to the next generation. Like Ikk.gamma./NEMO mutant mice and cells, cells and tissues from IP patients exhibit defective IKK.gamma./NEMO expression.

Detail Description Paragraph - DETX:

[0065] Like most other NF-.kappa.B-deficient cells, the IKK.gamma./NEMO-deficient keratinocytes are very sensitive to apoptosis; TUNEL assays revealed a large increase in the frequency of apoptotic cells in the epidermis of Ikk.gamma..sup.+/- mice. Increased apoptosis was not limited to the epidermis, but was also seen in the thymus and spleen. In all of these cases, apoptosis may be enhanced or driven by death cytokines produced by neighboring IKK.gamma./NEMO-positive cells. Many of the TUNEL-positive cells in the epidermis of Ikk.gamma..sup.+/- mice were clustered and appeared to be extruded from the epidermis by growth of the remaining normal cells. Most likely, this process contributes to the shedding of dead skin fragments and the eventual disappearance of the verrucous lesions. The third stage of the disease is characterized by streaks of hyperpigmentation and scarring (Landy and Donnai, J. Med. Genet. 30:53-59 [1993]; and Francis and Sybert, Semin. Cutan. Med. Surg. 16:54-60 [1997]). Histological examination reveals loss (incontinence) of melanin from basal cells of the epidermis and the appearance of free pigment deposits, or melanin-containing macrophages (Francis and Sybert, supra [1997]; and Scheuerle, J. Med. Genet. 77:201-218 [1998]). Although an understanding of the mechanism(s) is not necessary in order to use the present invention, the loss of pigment cells appears to be due to the apoptosis and necrosis of IKK.gamma./NEMO-deficient cells that release free melanin, some of which is ingested by macrophages. Eventually, all or most of the IKK.gamma./NEMO-deficient cells are eliminated through apoptosis and wherever possible, are replaced by surviving and proliferation-competent IKK.gamma./NEMO-expressing cells (See, FIG. 8). However, it is believed that some residual IKK.gamma./NEMO-deficient cells are capable of giving rise to another disease cycle, as has been occasionally observed (van Leeuwen et al., Pediatr. Dermatol. 17:70 [2000]). The importance of the **IKK** complex and NF-.kappa.B for prevention of apoptosis in humans, as well as mice is underscored by the contemplated association between **human** IP and IKK.gamma./NEMO-deficiency.

Detail Description Paragraph - DETX:

[0083] In these experiments, MEFs and ES cell extracts were examined. Wild-type and Ikk.gamma. mutant MEFs and ES cells were treated with TNF.alpha. (10 ng/ml) or IL-1 (20 ng/ml). Control cells (i.e., without TNF.alpha. or IL-1 treatment) were also included. At the times indicated in FIG. 1, Panel C, the **IKK** complex was immunoprecipitated from cell lysates with a polyclonal antibody directed against IKK.alpha. (M280, Santa Cruz), and its activity

measured by an immunocomplex **kinase** assay with GST-1.kappa.B.alpha. (1-54) as a substrate, as known in the art (See, Rothwarf et al., Nature 395:297-300 [1998]). Loading was normalized by immunoblotting with a monoclonal antibody against IKK.alpha. (1:500; Imgenex). Immunoblot analysis was conducted as known in the art and previously described (See, Hu et al., Science 284:316-320 [1999]). Electrophoretic mobility shift assays (EMSA) were performed as known in the art (See, Rothwarf et al., supra [1998]).

Detail Description Paragraph - DETX:

[0084] Consistent with the absence of IKK.gamma.NEMO, no **IKK** activity could be elicited in Ikk.gamma..sup.- ES cells or **murine** embryo fibroblasts (MEFs) stimulated with TNF.alpha. or IL-1 (See, FIG. 1, Panel C). Furthermore, no NF-.kappa.B DNA-binding activity could be induced in IKK.gamma./NEMO-deficient cells, and even basal NF-.kappa.B activity was dramatically reduced (See, FIG. 1, Panel D). Expression of the p50 and p65 subunits of NF-.kappa.B, however, was normal in IKK.gamma./NEMO-deficient MEFs. In addition, IKK.alpha. and IKK.beta. expression was found to be unaltered in all of the IKK.gamma./NEMO-deficient ES clones (the genotype of these cells is referred to herein as "Ikk.gamma..sup.-," regardless of origin).

Detail Description Paragraph - DETX:

[0102] These experiments were conducted in view of the similarity in the skin manifestations observed in Ikk.gamma..sup.+/- female mice and those associated with IP, as well as the identical chromosomal location of the IKK.gamma./NEMO and IP loci in humans. Thus, the level of **IKK** subunit expression was investigated using **human** skin biopsy materials. Immunohistochemical analysis revealed uniform expression of all three **IKK** subunits in epidermis of normal individuals as well as patients suffering from X-linked ichthyosis and psoriasis, as shown in FIG. 7, Panel A. IKK.alpha. and IKK.beta. were also invariantly and uniformly expressed in the epidermis of 11 different IP patients. However, scarce and sporadic expression of IKK.gamma./NEMO in a few cells within the epidermis was found in biopsies taken from lesions of 11 different IP patients (See, FIG. 7, Panel A, for data from one representative biopsy). Western blot analysis of two primary fibroblast cultures, established from an aborted male fetus, as well as a newborn which died within one day of birth (both of which were carried by the same IP mother), showed no expression if IKK.gamma./NEMO, but normal expression of IKK.alpha. and IKK.beta., as shown in FIG. 7, Panel B. Genomic linkage analysis confirmed that these fibroblasts contained the affected X chromosome (Roberts et al, Am. J. Med. Genet. 75:159-163 [1998]). Northern blot analysis revealed the absence of the IKK.gamma./NEMO gene product. Interestingly, the deceased IP newborn was found to have internal hemorrhage, excessive EMH, and granulocytic infiltrate in the lung, despite the absence of any identifiable infection (Roberts et al., supra [1998]).

Detail Description Paragraph - DETX:

[0108] In this example, biochemical analyses of wild type and IP fibroblasts

are described. **Human** wild type and IP fibroblasts were cultured in the presence of the cytokines TNF.alpha. and IL-1. A marked reduction in both **IKK** activation and NF-.kappa.B binding activity was observed in IP fibroblasts in which the putative zinc-finger was deleted (See, FIG. 13, Panels A & B). These effects were not due to a lack of p65 or p50 subunits, since both of these family members were expressed to similar levels in wild type and IP fibroblasts (See, FIG. 13, Panel C).

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TITLE: IKB kinase, subunits thereof, and methods of using same

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parent-patent 6242253 US non-provisional-of-provisional 60061470 19971009 US

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ABSTRACT:

The present invention provides an isolated nucleic acid molecules encoding I.kappa.B **kinase (IKK**) catalytic subunit polypeptides, which are associated with an **IKK** serine protein **kinase** that phosphorylates a protein (I.kappa.B) that inhibits the activity of the NF-.kappa.B transcription factor, vectors comprising such nucleic acid molecules and host cells containing such vectors. In addition, the invention provides nucleotide sequences that can bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful as probes or as antisense molecules. The invention also provides isolated **IKK** catalytic subunits, which can phosphorylate an I.kappa.B protein, and peptide portions of such **IKK** subunit. In addition, the invention provides anti-**IKK** antibodies, which specifically bind to an **IKK** complex or an **IKK** catalytic subunit, and **IKK**-binding fragments of such antibodies. The invention further provides methods of substantially purifying an **IKK** complex, methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** catalytic subunit with a second protein, and methods of identifying proteins that can interact with an **IKK** complex or an **IKK** catalytic subunit.

[0001] This appiication is based on, and claims the benefit of, U.S.

Provisional Application No. 60/061,470, filed Oct. 9, 1997, the entire contents of which is herein incorporated by reference.

---------- KWIC ---------

Abstract Paragraph - ABTX:

The present invention provides an isolated nucleic acid molecules encoding I.kappa.B **kinase (IKK**) catalytic subunit polypeptides, which are associated with an **IKK** serine protein **kinase** that phosphorylates a protein (I.kappa.B) that inhibits the activity of the NF-.kappa.B transcription factor, vectors comprising such nucleic acid molecules and host cells containing such vectors. In addition, the invention provides nucleotide sequences that can bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful as probes or as antisense molecules. The invention also provides isolated **IKK** catalytic subunits, which can phosphorylate an I.kappa.B protein, and peptide portions of such **IKK** subunit. In addition, the invention provides anti-**IKK** antibodies, which specifically bind to an **IKK** complex or an **IKK** catalytic subunit, and **IKK**-binding fragments of such antibodies. The invention further provides methods of substantially purifying an **IKK** complex, methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** catalytic subunit with a second protein, and methods of identifying proteins that can interact with an **IKK** complex or an **IKK** catalytic subunit.

Summary of Invention Paragraph - BSTX:

[0012] The present invention provides isolated nucleic acid molecules encoding full length **human** serine protein **kinases,** designated I.kappa.B **kinase (IKK**) subunits IKK.alpha. and IKK.beta.. The disclosed **IKK** subunits share substantial sequence homology and are activated in response to proinflammatory signals to phosphorylate proteins (I.kappa.B's) that inhibit the activity of the NF-.kappa.B transcription factor.

Summary of Invention Paragraph - BSTX:

[0015] The present invention also provides isolated full length **human IKK** subunits, which can phosphorylate an I.kappa.B protein. For example, the invention provides an IKK.alpha. polypeptide having the amino acid sequence shown as SEQ ID NO: 2, particularly the amino acid sequence comprising amino acids 1 to 31 at the N-terminus of the polypeptide of SEQ ID NO: 2. In addition, the invention provides an IKK.beta. polypeptide having the amino acid sequence shown as SEQ ID NO: 15. The invention also provides peptide portions of an **IKK** subunit, including, for example, peptide portions comprising one or more contiguous amino acids of the N-terminal amino acids shown as residues 1 to 31 in SEQ ID NO: 2. A peptide portion of an **IKK** subunit can comprise the **kinase** domain of the **IKK** subunit or can comprise a peptide useful for eliciting production of an antibody that specifically binds to an I.kappa.B **kinase or to the IKK** subunit. Accordingly, the invention also provides

anti-**IKK** antibodies that specifically bind to an **IKK** complex comprising an **IKK** subunit, particularly to the **IKK** subunit, for example, to an epitope comprising at least one of the amino acids shown as residues 1 to 31 of SEQ ID NO: 2, and also provides **IKK** subunit-binding fragments of such antibodies. In addition, the invention provides cell lines producing anti-**IKK** antibodies or **IKK**-binding fragments thereof.

Summary of Invention Paragraph - BSTX:

[0016] The invention also provides isolated I.kappa.B **kinase** complexes. As disclosed herein, an **IKK** complex can have an apparent molecular mass of about 900 kDa or about 300 kDa. An **IKK** complex is characterized, in part, in that it comprises an IKK.alpha. subunit, an IKK.beta. subunit, or both and can phosphorylate an I.kappa.B protein.

Summary of Invention Paragraph - BSTX:

[0017] The present invention further provides methods for isolating an **IKK** complex or an **IKK** subunit, as well as methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** subunit with a second protein that associates with the **IKK** in vitro or in vivo. Such a second protein can be, for example, another **IKK** subunit; an I.kappa.B protein, which is a substrate for **IKK** activity and is involved in a signal transduction pathway that results in the regulated expression of a gene; a protein that is upstream of the I.kappa.B **kinase** in a signal transduction pathway and regulates **IKK** activity; or a protein that acts as a regulatory subunit of the I.kappa.B **kinase or of an IKK** subunit and is necessary for full activation of the **IKK** complex. An agent that alters the association of an **IKK** subunit with a second protein can be, for example, a peptide, a polypeptide, a peptidomimetic or a small organic molecule. Such agents can be useful for modulating the level of phosphorylation of I.kappa.B in a cell, thereby modulating the activity of NF-.kappa.B in the cell and the expression of a gene regulated by NF-.kappa.B.

Summary of Invention Paragraph - BSTX:

[0018] The invention also provides methods of identifying proteins that can interact with an I.kappa.B **kinase,** including with an **IKK** subunit, such proteins which can be a downstream effector of the **IKK** such as a member of the I.kappa.B family of proteins or an upstream activator or a regulatory subunit of an **IKK**. Such proteins that interact with an **IKK** complex or the **IKK** subunit can be isolated, for example, by coprecipitation with the **IKK** or by using the **IKK** subunit as a ligand, and can be involved, for example, in tissue specific regulation of NF-.kappa.B activation and consequent tissue specific gene expression.

Brief Description of Drawings Paragraph - DRTX:

[0019] FIG. 1 shows a nucleotide sequence (SEQ ID NO: 1; lower case letter) and

deduced amino acid sequence (SEQ ID NO: 2; upper case letters) of full length **human** IKK.alpha. subunit of an **IKK** complex. Nucleotide positions are indicated to the right and left of the sequence; the "A" of the ATG encoding the initiator methionine is shown as position 1. Underlined amino acid residues indicate the peptide portions of the protein ("peptide 1" and "peptide 2") that were sequenced and used to design oligonucleotide probes. The asterisk indicates the sequence encoding the STOP codon.

Detail Description Paragraph - DETX:

[0022] The present invention provides isolated nucleic acid molecules encoding polypeptide subunits of **human** serine protein **kinase** complex, the I.kappa.B **kinase (IKK**), which is activated in response to proinflammatory signals and phosphorylates proteins (I.kappa.B's) that bind to and inhibit the activity of NF-.kappa.B transcription factors. For example, the invention provides an isolated nucleic acid molecule (SEQ ID NO: 1) encoding a full length **human** IKK.alpha. subunit having the amino acid sequence shown as SEQ ID NO: 2 (FIG. 1). In addition, the invention provides an isolated nucleic acid molecule (SEQ ID NO: 14; FIG. 2) encoding a full length **human** IKK.beta. subunit having the amino acid sequence shown as SEQ ID NO: 15 (FIG. 3).

Detail Description Paragraph - DETX:

[0024] IKK.alpha. and IKK.beta. have been designated **IKK** subunits because they are components of an approximately 900 kDa complex having I.kappa.B **kinase (IKK**) activity and because they share substantial nucleotide and amino acid sequence homology. As disclosed herein, IKK.alpha. and IKK.beta. are related members of a family of **IKK** catalytic subunits (see FIG. 3). The 900 kDa I.kappa.B **kinase** complex can be isolated in a single step, for example, by immunoprecipitation using an antibody specific for an **IKK** subunit or by using metal ion chelation chromatography methods (see Example IV). A 300 kDa **IKK** complex also can be isolated as disclosed herein and has **kinase** activity for an I.kappa.B substrate (see Example III).

Detail Description Paragraph - DETX:

[0031] The invention also provides nucleotide sequences that can specifically hybridize to a nucleic acid molecule of the invention. Such hybridizing nucleotide sequences are useful, for example, as probes, which can hybridize to a nucleic acid molecule encoding an **IKK** catalytic subunit and allow the identification of the nucleic acid molecule in a sample. A nucleotide sequence of the invention is characterized, in part, in that it is at least nine nucleotides in length, such sequences being particularly useful as primers for the polymerase chain reaction (PCR), and can be at least fourteen nucleotides in length or, if desired, at least seventeen nucleotides in length, such nucleotide sequences being particularly useful as hybridization probes, although such sequences also can be used for PCR. A nucleotide sequence of the invention can comprise at least six nucleotides 5' to nucleotide position 92 as shown in SEQ ID NO: 1 (FIG. 1), preferably at least nine nucleotides 5' to

position 92, or more as desired, where SEQ ID NO: 1 is shown in the conventional manner from the 5'-terminus (FIG. 1; upper left) to the 3'-terminus. Such nucleotide sequences of the invention are particularly useful in methods of diagnosing a pathology, for example, a **human** disease, characterized by aberrant **IKK** activity. For convenience, such nucleotide sequences can comprise a kit, which can be made commercially available and can provide a standardized diagnostic assay.

Detail Description Paragraph - DETX:

[0032] A nucleic acid molecule encoding an IKK.alpha. such as the nucleotide sequence shown in SEQ ID NO: 1 diverges from the sequence encoding the **mouse** homolog (GenBank Accession #U12473) in the region encoding amino acid 30. Thus, a nucleotide sequence comprising nucleotides 88 to 90 as shown in SEQ ID NO: 1, which encodes amino acid 30 of **human** IKK.alpha., can be particularly useful, for example, for identifying the presence of a nucleic acid molecule encoding a **human** IKK.alpha. in a sample. Furthermore, based on a comparison of SEQ ID NO: 1 with SEQ ID NO: 14, the skilled artisan readily can select nucleotide sequences that can hybridize with a nucleic acid molecule encoding a **human** IKK.alpha. or a **human** IKK.beta. or both by designing the sequence to contain conserved or non-conserved nucleotide sequences, as desired. For example, selection of a nucleotide sequence that is highly conserved among SEQ ID NO: 1 and SEQ ID NO: 14 can allow the identification of related members of the **IKK** subunit family of proteins. In comparison, selection of a nucleotide sequence that is present, for example, in SEQ ID NO: 14, but that is not present in SEQ ID NO: 1 or that shares only minimal homology can allow identification of the expression of SEQ ID NO: 14 in a cell, irrespective of whether SEQ ID NO: 1 also is expressed in the cell. It should be recognized, however, that a nucleotide sequence of the invention readily is identifiable in comparison to GenBank Accession #U12473 or #U22512 in that a nucleotide sequence of the invention is not the nucleotide sequence of GenBank Accession #U12473 or #U22512.

Detail Description Paragraph - DETX:

[0043] A "suicide" gene also can be incorporated into a vector so as to allow for selective inducible killing of a cell containing the gene. A gene such as the herpes simplex virus thymidine **kinase** gene (TK) can be used as a suicide gene to provide for inducible destruction of such cells. For example, where it is desired to terminate the expression of an introduced nucleic acid molecule encoding **IKK** or an antisense **IKK** subunit molecule in cells containing the nucleic acid molecule, the cells can be exposed to a drug such as acyclovir or gancyclovir, which can be administered to an individual.

Detail Description Paragraph - DETX:

[0063] The present invention provides an isolated I.kappa.B **kinase (IKK)**, including isolated full length **IKK** catalytic subunits. For example, the invention provides an isolated 300 kDa or 900 kDa complex, which comprises an

IKK.alpha. or an IKK.beta. subunit and has I.kappa.B **kinase** activity (see Examples I, III and IV). In addition, the invention provides is an isolated **human** IKK.alpha. catalytic subunit (SEQ ID NO: 2; Example II), which contains a previously undescribed N-terminal amino acid sequence and essentially the C-terminal region of **human** CHUK (Connelly and Marcu, supra, 1995) and phosphorylates I.kappa.B.alpha. on Ser-32 and Ser-36 and I.kappa.B.beta. on Ser-19 and Ser-23 (DiDonato et al., supra, 1996; see, also, Regnier et al., supra, 1997). The invention also provides an isolated IKK.beta. catalytic subunit (SEQ ID NO: 15; Example III), which shares greater than 50% amino acid sequence identity with IKK.alpha., including conserved homology in the **kinase** domain, helix-loop-helix domain and leucine zipper domain.

Detail Description Paragraph - DETX:

[0064] As used herein, the term "isolated," when used in reference to an I.kappa.B **kinase** complex or to an **IKK** catalytic subunit of the invention, means that the complex or the subunit is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with an **IKK** in a cell. An isolated 900 kDa I.kappa.B **kinase** complex or 300 kDa complex can be isolated, for example, by immunoprecipitation using an antibody that binds to an **IKK** catalytic subunit (see Examples III and IV). In addition, an isolated **IKK** subunit can be obtained, for example, by expression of a recombinant nucleic acid molecule such as SEQ ID NO: 1 or SEQ ID NO: 14, or can be isolated from a cell by a method comprising affinity chromatography using ATP or I.kappa.B as ligands (Example I) or using an anti-**IKK** subunit antibody. An isolated **IKK** complex or **IKK** subunit comprises at least 30% of the material in a sample, generally about 50% or 70% or 90% of a sample, and preferably about 95% or 98% of a sample, as described above with respect to nucleic acids.

Detail Description Paragraph - DETX:

[0065] The amino acid sequences for MEKK1 (GenBank Accession #U48596; locus RNU48596), PKR (GenBank Accession #M35663; locus HUMP68A) and CKII (GenBank Accession #M55268 J02924; locus HUMA1CKII) are different from the sequences of the **IKK** subunits disclosed herein (SEQ ID NO: 2 and SEQ ID NO: 15) and, therefore, are distinguishable from the present invention. In addition, a full length **human** IKK.alpha. of the invention is distinguishable from the partial **human** CHUK polypeptide sequence in that the partial **human** CHUK polypeptide (Connelly and Marcu, supra, 1995; GenBank Accession #22512) lacks amino acids 1 to 31 as shown in SEQ ID NO: 2. As disclosed herein, a polypeptide having the amino acid sequence of the partial **human** CHUK polypeptide does not have I.kappa.B **kinase** activity when expressed in a cell, indicating that some or all of amino acid residues 1 to 31 are essential for **kinase** activity.

Detail Description Paragraph - DETX:

[0066] A full length **IKK** catalytic subunit of the invention is exemplified by **human** IKK.alpha., which has an apparent molecular mass of about 85 kDa and phosphorylates I.kappa.B.alpha. on Ser-32 and Ser-36. An **IKK** catalytic

subunit of the invention also is exemplified by IKK.beta., which is an 87 kDa polypeptide that shares substantial amino acid sequence homology with IKK.alpha. (FIG. 3). As used herein, the term "full length," when used in reference to an **IKK** subunit of the invention, means a polypeptide having an amino acid sequence of an **IKK** subunit expressed normally in a cell. Such a normally expressed **IKK** polypeptide begins with a methionine residue at its N-terminus (Met-1; FIG. 3), the Met-1 being encoded by the initiator ATG (AUG) codon, and ends as a result of the termination of translation due to the presence of a STOP codon. A full length **human IKK** catalytic subunit can be a native **IKK** polypeptide, which is isolated from a cell, or can be produced using recombinant DNA methods such as by expressing the nucleic acid molecule shown as SEQ ID NO: 1 or SEQ ID NO: 14.

Detail Description Paragraph - DETX:

[0072] A peptide portion of an **IKK** subunit can comprise the **kinase** domain of the **IKK** subunit and, therefore, can have the ability to phosphorylate an I.kappa.B protein. For example, a peptide portion of SEQ ID NO: 2 comprising amino acids 15 to 301 has the characteristics of a serine-threonine protein **kinase** domain (Hanks and Quinn, Meth. Enzymol. 200:38-62 (1991), which is incorporated herein by reference). Such a peptide portion of an **IKK** subunit can be examined for **kinase** activity by determining that it can phosphorylate I.kappa.B.alpha. at Ser-32 and Ser-36 or I.kappa.B.beta. at Ser-19 and Ser-23, using methods as disclosed herein. In addition, a peptide portion of an **IKK** subunit can comprise an immunogenic amino acid sequence of the polypeptide and, therefore, can be useful for eliciting production of an antibody that can specifically bind the **IKK** subunit or to an **IKK** complex comprising the subunit, particularly to an epitope comprising amino acid residue 30 as shown in SEQ ID NO: 2 or to an epitope of SEQ ID NO: 15, provided said epitope is not present in a CHUK protein. Accordingly, the invention also provides anti-**IKK** antibodies, which specifically bind to an epitope of an **IKK** complex, particularly an **IKK** catalytic subunit, and to **IKK** subunit binding fragments of such antibodies. In addition, the invention provides cell lines producing anti-**IKK** antibodies or **IKK**-binding fragments of such antibodies.

Detail Description Paragraph - DETX:

[0076] An anti-**IKK** antibody of the invention can be raised using an isolated **IKK** subunit or a peptide portion thereof and can bind to a free, uncomplexed form of **IKK** subunit or can bind to **IKK** subunit when it is associated with a 300 kDa or 900 kDa **IKK** complex. In addition, an anti-**IKK** antibody of the invention can be raised against an isolated 300 kDa or 900 kDa I.kappa.B **kinase** complex, which can be obtained as disclosed herein. For convenience, an antibody of the invention is referred to generally herein as an "anti-I.kappa.B **kinase** antibody" or an "anti-**IKK** antibody." However, the skilled recognize that the various antibodies of the invention will have unique antigenic specificities, for example, for a free or complexed **IKK** subunit, or both, or for a 300 kDa or 900 kDa I.kappa.B **kinase** complex, or both.

Detail Description Paragraph - DETX:

[0077] Anti-**IKK** antibodies can be raised using as an immunogen an isolated full length **IKK** catalytic subunit, which can be prepared from natural sources or produced recombinantly, or a peptide portion of an **IKK** subunit as defined herein, including synthetic peptides as described above. A non-immunogenic peptide portion of an **IKK** catalytic subunit can be made immunogenic by coupling the hapten to a carrier molecule such bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane, supra, 1988). It is recognized that, due to the apparently high amino acid sequence identity of the full length **human** IKK.alpha. and **mouse** CHUK, the amino acid sequences of IKK.alpha. polypeptides, as well as IKK.beta. polypeptides, likely are highly conserved among species, particularly among mammalian species. However, antibodies to highly conserved proteins have been raised successfully, for example, in chickens. Such a method can be used to obtain an antibody to an **IKK** subunit, if desired.

Detail Description Paragraph - DETX:

[0078] Particularly useful antibodies of the invention include antibodies that bind with the free, but not the complexed, form of an **IKK** subunit or, alternatively, with the complexed, but not free, form of an **IKK** subunit. Antibodies of the invention also include antibodies that bind with the 300 kDa I.kappa.B **kinase** complex or the 900 kDa I.kappa.B **kinase** complex or both. It should be recognized, however, that an antibody specific for the 300 kDa or 900 kDa I.kappa.B **kinase** complex need not recognize an **IKK** subunit epitope in order to be encompassed within the claimed invention, since, prior to the present disclosure, the 300 kDa and 900 kDa **IKK** complexes were not known (see DiDonato et al., Nature 388:548-554 (1997)).

Detail Description Paragraph - DETX:

[0079] Antibodies of the invention that bind to an activated **IKK** but not to an inactive **IKK**, and, conversely, those that bind to an inactive form of the **kinase** but not to the activated form also are particularly useful. For example, an **IKK** can be activated by phosphorylation of an **IKK** subunit and, therefore, an antibody that recognizes the phosphorylated form of the **IKK**, but that does not bind to the unphosphorylated form can be obtained. In addition, **IKK** can be activated by release of a regulatory subunit and, therefore, an antibody that recognizes a form of the **IKK** complex that is not bound to the regulatory subunit can be obtained. Such antibodies are useful for identifying the presence of active **IKK** in a cell.

Detail Description Paragraph - DETX:

[0080] An anti-**IKK** antibody is useful, for example, for determining the presence or level of an **iKK or of an IKK** subunit in a tissue sample, which can

be a lysate or a histological section. The identification of the presence or level of an **IKK or an IKK** subunit in the sample can be made using well known immunoassay and immunohistochemical methods (Harlow and Lane, supra, 1988). An anti-**IKK** antibody also can be used to substantially purify an I.kappa.B **kinase or an IKK** subunit from a sample. In addition, an anti-**IKK** antibody can be used in a screening assay to identify agents that alter the activity of an I.kappa.B **kinase**.

Detail Description Paragraph - DETX:

[0081] A kit incorporating an anti-**IKK** antibody, which can be specific for the active or inactive form of I.kappa.B **kinase** or can bind to an **IKK** complex or to an **IKK** subunit, regardless of the activity state, can be particularly useful. Such a kit can contain, in addition to an anti-**IKK** antibody, a reaction cocktail that provides the proper conditions for performing the assay, control samples that contain known amounts of an **IKK or IKK** subunit and, if desired, a second antibody specific for the anti-**IKK** antibody. Such an assay also should include a simple method for detecting the presence or amount of an **IKK or an IKK** subunit in a sample that is bound to the anti-**IKK** antibody.

Detail Description Paragraph - DETX:

[0083] Following contact, for example, of a labeled antibody with a sample such as a tissue homogenate or a histological section of a tissue, specifically bound labeled antibody can be identified by detecting the particular moiety. Alternatively, a labeled second antibody can be used to identify specific binding of an unlabeled anti-**IKK** antibody. A second antibody generally will be specific for the particular class of the first antibody. For example, if an anti-I.kappa.B **kinase** antibody is of the IgG class, a second antibody will be an anti-IgG antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labeled using a detectable moiety as described above. When a sample is labeled using a second antibody, the sample is first contacted with a first antibody, which is an anti-**IKK** antibody, then the sample is contacted with the labeled second antibody, which specifically binds to the anti-**IKK** antibody and results in a labeled sample.

Detail Description Paragraph - DETX:

[0084] Methods for raising polyclonal antibodies, for example, in a rabbit, goat, **mouse** or other mammal, are well known in the art (see Example V). In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (Harlow and Lane, supra, 1988). Essentially, spleen cells from a **mouse** immunized with an **IKK** complex or an **IKK** subunit or peptide portion thereof can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using a labeled **IKK** subunit to identify clones that secrete anti-**IKK** monoclonal antibodies. Hybridomas expressing anti-**IKK** monoclonal antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies, which are useful, for

example, for preparing standardized kits as described above. Similarly, a recombinant phage that expresses, for example, a single chain anti-**IKK** also provides a monoclonal antibody that can used for preparing standardized kits.

Detail Description Paragraph - DETX:

[0085] A monoclonal anti-**IKK** antibody can be used to prepare anti-idiotypic antibodies, which present an epitope that mimics the epitope recognized by the monoclonal antibody used to prepare the anti-idiotypic antibodies. Where the epitope to which the monoclonal antibody includes, for example, a portion of the **IKK** catalytic subunit **kinase** domain, the anti-idiotypic antibody can act as a competitor of I.kappa.B and, therefore, can be useful for reducing the level of phosphorylation of I.kappa.B and, consequently, the activity of NF-.kappa.B.

Detail Description Paragraph - DETX:

[0086] The present invention further provides methods of identifying an agent that can alter the association of an **IKK** catalytic subunit with a second protein, which can be an upstream activator, a downstream effector such as I.kappa.B, an interacting regulatory protein of the **IKK** subunit, or an interacting subunit associated with the 300 kDa or 900 kDa I.kappa.B **kinase** complex. As used herein, the term "associate" or "association," when used in reference to an **IKK** subunit and a second protein means that the **IKK** subunit and the second protein have a binding affinity for each other such that they form a bound complex in vivo or in vitro, including in a cell in culture or in a reaction comprising substantially purified reagents. For convenience, the term "bind" or "interact" is used interchangeably with the term "associate."

Detail Description Paragraph - DETX:

[0088] As used herein, the term "second protein" refers to a protein that specifically associates with an **IKK** subunit ("first protein"). Such a second protein is exemplified herein by I.kappa.B proteins, including I.kappa.B.alpha. and I.kappa.B.beta., which are substrates for I.kappa.B **kinase** activity and are downstream of the I.kappa.B **kinase** in a signal transduction pathway that results in the regulated expression of a gene. In addition, such second proteins are exemplified by the proteins that, together with the **IKK** subunits, form a 300 kDa or 900 kDa I.kappa.B **kinase** complex, which coimmunoprecipitates using an anti-**IKK** antibody (see Example IV). Furthermore, since **IKK** subunits such as IKK.alpha. and IKK.beta. interact with each other to form homodimers or heterodimers, a second protein also can be a second **IKK** subunit, which can be the same as or different from the "first" protein.

Detail Description Paragraph - DETX:

[0089] Agents that alter the association of an **IKK** catalytic subunit and a second protein such as I.kappa.B protein or an **IKK** regulatory subunit can be extremely valuable, for example, for limiting excessive cytokine expression as

occurs in an acute phase response by preventing the activation of NF-.kappa.B, thereby preventing NF-.kappa.B mediated induction of cytokine gene expression. Where, in a drug screening assay of the invention, the second protein is an I.kappa.B, the **IKK** subunit can be any protein involved in I.kappa.B **kinase** activity, including, for example, **mouse** CHUK (Connelly and Marcu, supra, 1995; GenBank Accession #12473), which, prior to the present disclosure, was not known to have the ability to associate with I.kappa.B or to have I.kappa.B **kinase** activity.

Detail Description Paragraph - DETX:

[0090] In addition, a second protein can be a protein that is upstream of I.kappa.B **kinase** in a signal transduction pathway and associates with the **IKK** complex, particularly with an **IKK** catalytic subunit of the **IKK** complex. Such a second protein, which can be an upstream activator of the I.kappa.B **kinase**, can be identified using routine methods for identifying protein-protein interactions as disclosed herein. Such second proteins can be, for example, MEKK1 or PKR or CKII, each of which has been reported to be involved in a pathway leading to phosphorylation of I.kappa.B and activation of NF-.kappa.B, but neither of which has the characteristics expected of the common I.kappa.B **kinase** present at the point where the various NF-.kappa.B activation pathways converge (see, for example, Lee et al., supra, 1997), or can be the NF-.kappa.B-inducing **kinase** (NIK), which reportedly is upstream from **IKK** in an NF-.kappa.B activation pathway (Regnier et al., supra, 1997; Malinin et al., Nature 385:540-544 (1997)).

Detail Description Paragraph - DETX:

[0092] As disclosed herein, two copurifying proteins were isolated by ATP and I.kappa.B affinity chromatography and identified by SDS-PAGE (Example I). Partial amino acid sequences were determined and cDNA molecules encoding the proteins were obtained (see Examples I, II and III). One of the proteins has an apparent molecular mass of 85 kDa. Expression in a cell of a cDNA molecule encoding the 85 kDa protein resulted in increased NF-.kappa.B activity following cytokine induction as compared to control cells, whereas expression of the antisense of this cDNA decreased the basal NF-.kappa.B activity in the cells and prevented cytokine induction of NF-.kappa.B activity. Immunoprecipitation of the 85 kDa protein resulted in isolation of the **IKK** complex, the **kinase** activity of which was stimulated rapidly in response to TNF or to IL-1. Based on these functional analyses, the 85 kDa protein was determined to be a component of the 900 kDa I.kappa.B **kinase** complex and has been designated IKK.alpha. (SEQ ID NO: 2). The second protein, which copurified with the 85 kDa I.kappa.B **kinase**, has an apparent molecular mass of 87 kDa and shares greater than 50% amino acid sequence identity with IKK.alpha. and has been designated IKK.beta. (SEQ ID NO: 15).

Detail Description Paragraph - DETX:

[0093] The ability of the 85 kDa and 87 kDa **IKK** subunits to associate with

other proteins such as a regulatory subunit as well as with I.kappa.B is suggested, for example, by the presence in the I.kappa.B **kinase** of two different protein binding domains, a helix-loop-helix domain and a leucine zipper domain (see Connelly and Marcu, supra, 1995; see, also, FIG. 3). While the leucine zipper motif mediates homotypic and heterotypic interactions between IKK.alpha. and IKK.beta., the helix-loop-helix motif serves as a binding site for regulatory proteins necessary for I.kappa.B **kinase** activation.

Detail Description Paragraph - DETX:

[0095] A screening assay of the invention also is useful for identifying agents that directly alter the activity of an **IKK**. While such an agent can act, for example, by altering the association of an **IKK** complex or **IKK** catalytic subunit with a second protein, the agent also can act directly as a specific activator or inhibitor of **IKK** activity. Specific protein **kinase** inhibitors include, for example, staurosporin, the heat stable inhibitor of cAMP-dependent protein **kinase,** and the MLCK inhibitor, which are known in the art and commercially available. A library of molecules based, generally, on such inhibitors or on ATP or adenosine can be screened using an assay of the invention to obtain agents that desirably modulate the activity of an **IKK** complex or an **IKK** subunit.

Detail Description Paragraph - DETX:

[0096] As disclosed herein, **IKK** activity can be measured by identifying phosphorylation, for example, of I.kappa.B.alpha., either directly or using an antibody specific for the Ser-32 and Ser-36 phosphorylated form of I.kappa.B.alpha.. An antibody that binds to I.kappa.B.alpha. that is phosphorylated on Ser-32, for example, can be purchased from a commercial source (New England Biolabs; Beverly Mass.). Cultured cells can be exposed to various agents suspected of having the ability to directly alter **IKK** activity, then aliquots of the cells either are collected or are treated with a proinflammatory stimulus such as a cytokine, and collected. The collected cells are lysed and the **kinase** is immunoprecipitated using an anti-**IKK** antibody. A substrate such as I.kappa.B.alpha. or I.kappa.B.beta. is added to the immunocomplex and the ability of the **IKK** to phosphorylate the substrate is determined as described above. If desired, the anti-**IKK** antibody first can be coated onto a plastic surface such as in 96 well plates, then the cell lysate is added to the wells under conditions that allow binding of **IKK** by the antibody. Following washing of the wells, **IKK** activity is measured as described above. Such a method is extremely rapid and provides the additional advantage that it can be automated for high through-put assays.

Detail Description Paragraph - DETX:

[0103] The specific cellular target upon which a cytokine restraining agent acts has not been reported. However, the myriad of pathologic effects ameliorated by such agents are similar to various pathologies associated with aberrant NF-.kappa.B activity, suggesting that cytokine restraining agents may

target an effector molecule in a NF-.kappa.B signal transduction pathway. Thus, one potential target of a cytokine restraining agent can be an I.kappa.B **kinase, particularly an IKK** catalytic subunit of the **kinase**. Accordingly, a screening assay of the invention can be used to determine whether a cytokine restraining agent alters the activity of I.kappa.B **kinase** or alters the association of an **IKK** and a second protein such as I.kappa.B. If it is determined that a cytokine restraining agent has such an effect, the screening assay then can be used to screen a library of cytokine regulatory agents to identify those having desirable characteristics, such as those having the highest affinity for the **IKK**.

Detail Description Paragraph - DETX:

[0105] The skilled artisan will recognize that a ligand such as ATP or an I.kappa.B or an anti-**IKK** antibody also can be immobilized on various other matrices, including, for example, on magnetic beads, which provide a rapid and simple method of obtaining a fraction containing an ATP-or an I.kappa.B-bound **IKK** complex or **IKK** subunit or an anti-I.kappa.B **kinase**-bound **IKK** from the remainder of the sample. Methods for immobilizing a ligand such as ATP or an I.kappa.B or an antibody are well known in the art (Haystead et al., Eur. J. Biochem. 214:459-467 (1993), which is incorporated herein by reference; see, also, Hermanson, supra, 1996). Similarly, the artisan will recognize that a sample containing an **IKK** complex or an **IKK** subunit can be a cell, tissue or organ sample, which is obtained from an animal, including a mammal such as a **human,** and prepared as a lysate; or can be a bacterial, insect, yeast or mammalian cell lysate, in which an **IKK** catalytic subunit is expressed from a recombinant nucleic acid molecule. As disclosed herein, a recombinantly expressed IKK.alpha. or IKK.beta. such as a tagged IKK.alpha. or IKK.beta. associates into an active 300 kDa and 900 kDa **IKK** complex (see Examples III and IV).

Detail Description Paragraph - DETX:

Identification and Characterization of a **Human** I.kappa.B **Kinase** Complex And **IKK** Subunits

Detail Description Paragraph - DETX:

[0123] **Kinase** assays were performed using GST fusion proteins containing amino acid residues 1 to 54 of I.kappa.B. The fusion proteins were linked to glutathione SEPHAROSE and the beads were used directly in the assays. At earlier stages in the purification of the **IKK** activity, the beads were washed prior to loading onto the gel to minimize contributions from other proteins. In some of the later characterization of highly purified material, soluble fusion protein was used.

Detail Description Paragraph - DETX:

[0124] Three distinct substrates for the **IKK** activity were used: 1) substrate "WT" contained amino acid residues 1 to 54 of I.kappa.B.alpha.; 2) substrate "AA" contained amino acid residues 1 to 54 of I.kappa.B.alpha., except that Ser-32 (S32) and S36 were replaced with Ala-32 (A32) and A36, respectively; and 3) substrate "TT" contained amino acid residues 1 to 54 of I.kappa.B.alpha., except that S32 and S36 were replaced with Thr-32 (T32) and T36, respectively (DiDonato et al., Mol. Cell. Biol. 16:1295-1304 (1996)). Each substrate was expressed as a GST fusion protein. The physiologic, inducible I.kappa.B **kinase** is specific for S32 and S36 (WT) in I.kappa.B.alpha., but does not recognize the TT or AA mutants (DiDonato et al., Mol. Cell. Biol. 16:1295-1304 (1996)).

Detail Description Paragraph - DETX:

[0132] Supernatant was collected and centrifuged at 38,000 rpm for 80 min in a Beckman 50.1 Ti rotor at 4.degree. C. The supernatant (S100 fraction) was quick frozen in liquid nitrogen and stored at .sup.-80.degree. C. Small aliquots of S100 material, prepared from either unstimulated HeLa cells or from TNF.alpha. stimulated cells, were purified in a single passage over a SUPEROSE 6 gel filtration column (1.0.times.30 cm; Pharmacia; Uppsalla Sweden) equilibrated in Buffer A containing 0.1% Brij-35 and 300 mM NaCl and eluted at a flow rate of 0.3 ml/min. 0.6 ml fractions were collected and **kinase** assays were performed on an aliquot of each fraction. The high molecular weight material (fractions 16-20) contained TNF.alpha.-inducible **IKK** activity, which is specific for the WT substrate.

Detail Description Paragraph - DETX:

[0133] 110 ml of S100 material (900 mg of protein; Bio-Rad Protein Assay) was pumped onto a Q-SEPHAROSE FAST FLOW column (56 ml bed volume, 2.6 cm ID) equilibrated at 2 ml/min with Buffer A containing 0.1% Brij-35. After the sample was loaded, the column was washed with 100 ml of Buffer A containing 0.1% Brij-35 and 100 mM NaCl, then a linear NaCl gradient was run from 100-300 mM. The gradient volume was 500 ml and the flow rate was 2 ml/min. Ten ml fractions were collected and the **kinase** assay was performed on those fractions that eluted during the gradient. Fractions corresponding to the TNF.alpha.-inducible **IKK** activity (fractions 30-42; i.e., 20-32 of the gradient portion) were pooled. The pooled material contained 40 mg of protein.

Detail Description Paragraph - DETX:

[0140] Since the 85 kDa IKK.alpha. band identified by the **kinase** assay following the above procedure contained only about 10% of the total purified protein, three additional criteria were used to confirm that the identified band was an intrinsic component of the **IKK** complex.

Detail Description Paragraph - DETX:

[0141] In one procedure, the elution profile of the SUPEROSE 6 column was

analyzed by silver stained 8% SDS-PAGE gels, then compared to the **kinase** activity profile. For this analysis, 0.3 ml fractions were collected from the SUPEROSE 6 column, then separated by 8% SDS-PAGE and silver stained. This comparison confirmed that a single band of 85 kDa correlated precisely with the elution of **IKK** activity.

Detail Description Paragraph - DETX:

[0146] In a third procedure, purified **IKK** was treated with excess phosphatase, which inactivates the **IKK,** then reactivated by addition of a semi-purified HeLa extract. Phosphatase inactivation was performed by adding excess protein phosphatase 2A catalytic domain (PP2A) to purified I.kappa.B **kinase** in 50 mM Tris (pH 7.6), 50 mM NaCl, 1 mM MgCl.sub.2, then equilibrating the reaction for 60 min at 30.degree. C. 1.25 .mu.M okadaic acid was added to completely inactivate the phosphatase and the phosphatase inactivated material was used in standard **kinase** assays and to perform the reactivation and phosphorylation procedure.

Detail Description Paragraph - DETX:

[0147] Cytoplasmic extract was prepared using HeLa S3 cells. The cells were stimulated with TNF.alpha. for 5 min, then harvested in lysis buffer containing 0.1% NP-40 and 0.15 M NaCl. Reactivation was performed at 30.degree. C. in **kinase** buffer for 60 min in the absence of (.gamma.-.sup.32P)ATP. Samples containing only cold ATP were used for **kinase** activity assays. Reactivation by the HeLa cell extract was performed in the presence of (.gamma.-.sup.32P)ATP, then the sample was separated by 8% SDS-PAGE and examined by autoradiography. A band of approximately 86 kDa was phosphorylated in the reactivated material and, associated with the reactivation procedure, was restoration of the **IKK** activity.

Detail Description Paragraph - DETX:

[0172] Comparison of the amino acid sequences of IKK.alpha. and IKK.beta. revealed greater than 50% amino acid identity (FIG. 3). In addition, SEQ ID NO: 15 contains a **kinase** domain, which shares 65% amino acid identity with IKK.alpha., a leucine zipper and a helix-loop-helix domain. Based on the sequence homology and domain structure, the polypeptide (SEQ ID NO: 15) was determined to be a member of the **IKK** catalytic subunit family of proteins with IKK.alpha. and, therefore, was designated IKK.beta..

Detail Description Paragraph - DETX:

[0178] The response of IKK.beta.-associated **kinase** activity to various stimuli also was examined in HeLa cells transiently transfected with the HA-IKK.beta. expression vector. After 24 hr, the cells were stimulated with either 10 ng/ml IL-1, 20 ng/ml TNF or 100 ng/ml TPA, then HA-IKK.beta. immune complexes were isolated by immunoprecipitation and **IKK** activity was measured. TNF and IL-1

potently stimulated IKK.beta.-associated **kinase** activity, whereas the response to TPA was weaker. The kinetics of IKK.beta. activation by either TNF or IL-1 essentially were identical to the kinetics of activation of the IKK.alpha.-associated I.kappa.B **kinase** measured by a similar protocol.

Detail Description Paragraph - DETX:

[0182] The levels of **IKK** activities associated with IKK.alpha. and IKK.beta. were compared more precisely by transfecting 293 cells with increasing amounts of HA-IKK.alpha. or HA-IKK.beta. expression vectors (0.1 to 0.5 .mu.g/10.sup.6 cells) and determining the **kinase** activities associated with the two proteins in cell lysates prepared before or after TNF stimulation (20 ng/ml, 5 min); GST-I.kappa.B.alpha.(1-54) was used as substrate. The level of expression of each protein was determined by immunoblot analysis and used to calculate the relative levels of specific **IKK** activity.

Detail Description Paragraph - DETX:

[0189] The relative contribution of IKK.alpha. and IKk.beta. to **IKK** activity was examined by constructing mutant subunits in which the lysine (K) codon present at position 44 of each subunit was substituted with a codon for either methionine (M) or alanine (A) codon, respectively. Similar mutations in other protein **kinases** render the enzymes defective in binding ATP and, therefore, catalytically inactive (Taylor et al., Ann. Rev. Cell Biol. 8:429-462 (1992)). The activity of the **IKK** mutants was compared to the activity of their wild type (wt) counterparts by cell-free translation in reticulocyte lysates using GST-I.kappa.B.alpha. (1-54) as a substrate. Translation of IKK.alpha. (KM) resulted in formation of I.kappa.B **kinase** having only slightly less activity than the **IKK** formed by translation of wt IKK.alpha.. In comparison, translation of IKK.beta. (KA) did not generate **IKK** activity. Translation of wt IKK.beta. generated I.kappa.B **kinase** activity as expected.

Detail Description Paragraph - DETX:

[0194] Expression of wt HA-IKK.alpha. generated substantial **IKK** activity that was isolated by immunoprecipitation with anti-HA, whereas very little **IKK** activity was generated in cells transfected with either the HA-IKK.alpha. (LZ).sup.- or HA-IKK.alpha. (HLH).sup.- mutant. Coexpression of the mutant **IKK** subunits with Flag-IKK.beta. resulted in a substantial increase in the **IKK** activity isolated by immunoprecipitation of HA-IKK.beta., but had no effect on the very low activity that coprecipitated with HA-IKK.alpha. (LZ).sup.-. However, coexpression of Flag-IKK.beta. did stimulate the low level of **IKK** activity associated with HA-IKK.alpha. (HLH).sup.-. Probing of the HA immune complexes with anti-Flag(M2) antibodies indicated that both wt HA-IKK.alpha. and HA-IKK.alpha. (HLH).sup.- associated with similar amounts of Flag-IKK.beta., but that the HA-IKK.alpha. (LZ) mutant did not associate with Flag-IKK.beta.. These results indicate that the lower I.kappa.B **kinase** activity associated with the IKK.alpha. (LZ).sup.- mutant is due to a defect in its ability to interact with IKK.beta.. The lower I.kappa.B **kinase** activity

of the IKK.alpha. (HLH).sup.- mutant, on the other hand, likely is due to a defect in the ability to interact with a second, undefined protein, since the HLH mutant can interact with IKK.beta..

Detail Description Paragraph - DETX:

[0202] pRC-HF-IKK.alpha. was transfected into **human** embryonic kidney 293 cells and transfected cells were selected for growth in the presence of G418. A low basal level of **IKK** activity was detected in cells expressing HF-IKK.alpha. and **IKK** activity increased several fold when the cells were treated with TNF.alpha.. This result indicates that the HF-IKK.alpha. expression in 293 cells is associated with **IKK** activity in the cells and that such **IKK** activity is inducible in response to TNF.alpha..

Detail Description Paragraph - DETX:

[0212] The second protein, which can be I.kappa.B or a protein that copurifies with **IKK** subunit as part of the 900 kDa I.kappa.B **kinase,** for example, can be detectably labeled with a moiety such as a fluorescent molecule or a radiolabel (Hermanson, supra, 1996), then contacted in solution with the immobilized **IKK** subunit under conditions as described in Example I, which allow I.kappa.B to specifically associate with the **IKK** subunit. Preferably, the reactions are performed in 96 well plates, which allow automated reading of the reactions. Various agents such as drugs then are screened for the ability to alter the association of the **IKK** subunit and I.kappa.B.

Claims Text - CLTX:

1. An isolated nucleic acid molecule, comprising a nucleotide sequence encoding an I.kappa.B **kinase (IKK)** subunit, IKK.beta., which phosphorylates the inhibitor of NF-.kappa.B (I.kappa.B.alpha.) on serine-32 and serine-36 and has an apparent molecular mass of about 87 kiloDaltons; or a nucleotide sequence complementary thereto.

Claims Text - CLTX:

10. An isolated I.kappa.B **kinase (IKK)** subunit, IKK.beta., wherein said IKK.beta. phosphorylates serine-32 and serine-36 of I.kappa.B.alpha. and has an apparent molecular mass of 87 kiloDaltons as determined by SDS-polyacrylamide gel electrophoresis in an 8% gel under reducing conditions.

Claims Text - CLTX:

16. A method of identifying an agent that modulates the specific association of a polypeptide of an I.kappa.B **kinase (IKK)** subunit and a second protein, comprising the steps of: a) contacting the **IKK** subunit and the second protein, under conditions suitable for the specific association of said **IKK** subunit and

said second protein, with an agent suspected of being able to modulate said specific association; and b) detecting an altered association of said **IKK** subunit and said second protein in the presence of said agent, wherein said altered association identifies an agent that modulates the specific association of said **IKK** subunit and said second protein.

Claims Text - CLTX:

29. A method for identifying an agent that alters I.kappa.B **kinase (IKK)** activity, comprising the steps of: a) incubating an isolated composition having **IKK** activity with an agent suspected of being able to alter said **IKK** activity; and b) determining altered **IKK** activity of said composition in the presence of said agent, wherein said altered **IKK** activity identifies an agent that alters said **IKK** activity of said composition.

Claims Text - CLTX:

34. A method of obtaining isolated I.kappa.B **kinase (IKK)** from a sample containing the **IKK,** comprising the steps of: a) contacting the sample containing the **IKK** with an antibody the specifically binds an epitope of the **IKK**; and b) obtaining isolated **IKK** bound to said antibody.

Claims Text - CLTX:

40. A method of modulating NF-.kappa.B activity in a cell, comprising contacting the cell with an agent that alters the association of an I.kappa.B **kinase (IKK) or an IKK** catalytic subunit and a second protein.

US-PAT-NO: 6365366

DOCUMENT-IDENTIFIER: US 6365366 B1

TITLE: T2k kinase assays

DATE-ISSUED: April 2, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Cao; Zhaodan	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 524435

DATE FILED: March 13, 2000

US-CL-CURRENT: 435/21; 435/15 ; 435/4 ; 435/7.1 ; 435/968 ; 530/300

ABSTRACT:

T2K kinase activity is detected by forming a mixture of a T2K kinase and a substrate; incubating the mixture under conditions whereby the kinase phosphorylates the substrate at a first rate; and detecting the first rate as an indication of the kinase activity. The substrate comprises SX.sub.1 X.sub.2 X.sub.3 SX.sub.4 (SEQ ID NO:1) wherein X.sub.1 and X.sub.4 are aliphatic residues and both of the S residues are targets of the kinase, and especially, IKK.alpha. or IKK.beta.. In another embodiment, the mixture substrate comprises a particular IL-1 or TNF signaling cascade component. The mixture may be used to screen for agents which modulate the activity of the kinase, e.g. as an immuno-chemiluminescent assay.

17 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Brief Summary Text - BSTX:

Inflammatory cytokines IL-1 and TNF exert diverse biological activities by altering gene expression in the cells, a function mediated in part by transcription factor NF-.kappa.B. In unstimulated cells, NF-.kappa.B proteins form a complex with inhibitory molecules, the I.kappa.B proteins, and are rendered inactive in the cytoplasm. In response to cytokines and other stimuli, the I.kappa.B proteins are phosphorylated on specific serine residues. Delineating TNF and IL-1 signaling pathways for NF-.kappa.B activation has

implicated the TRAF molecules as converging point for different cytokines, with TRAF2 being involved in TNF- and TRAF6 in IL-1-induced NF-.kappa.B activation. We previously disclosed a family of I.kappa.B **kinases** including a TRAF2-associated **kinase** activity (designated T2K) and the translation product of the KIAA0151 gene product (also known as **IKKi** and IKK.epsilon.) that phosphorylates the I.kappa.B molecules on the specific regulatory serine residues. We have now found that T2K and **IKKi** in fact have alternative substrate specificities and alternative physiologically relevant substrate targets, particularly IKK.alpha. and IKK.beta. peptide substrates. We disclose here materials and methods for assaying for these novel specificities.

Brief Summary Text - BSTX:

IKKi is described by Nagase et al., DNA Res.,1995, 2, 167-174; Genbank Accession Nos. D63485 and BAA09772 (human); **and by** Shimada et al., Int Immunol. 1999 Aug;11(8):1357-62; Genbank Accession Nos.AB016590 and BAA85155.1 (human); A**B0165**89 and BAA85154.1 (mouse).

Brief Summary Text - BSTX:

The invention provides a method for detecting **kinase** activity comprising the steps of (a) forming a mixture comprising an active T2K **kinase** and a T2K substrate; (b) incubating the mixture under conditions whereby the **kinase** phosphorylates the substrate at a first rate; and (c) detecting the first rate as an indication of the **kinase** activity. As used herein, the term T2K **kinase** describes the two related natural proteins T2K and natural **IKKi,** however insubstantial variants such as minor truncations, etc. which retain substantially the same **kinase** activity and specificity of the native proteins are clearly equivalents in the disclosed assays. The T2K **kinases** may be produced recombinantly and/or isolated from any convenient source, particularly **human or murine** cells, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKKi at 2**0 .m**u.g/ml in PBS.

US-PAT-NO: 6258579

DOCUMENT-IDENTIFIER: US 6258579 B1

TITLE: Stimulus-inducible protein kinase complex and methods of use therefor

DATE-ISSUED: July 10, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mercurio; Frank	San Diego	CA	N/A	N/A
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APPL-NO: 08/ 910820

DATE FILED: August 13, 1997

PARENT-CASE:

CROSS-REFERENCE TO PRIOR APPLICATION This application is a continuation-in-part of U.S. patent application Ser. No. 08/697,393, filed Aug. 26, 1996 now U.S. Pat. No. 5,972,674.

US-CL-CURRENT: 435/194

ABSTRACT:

Compositions and methods are provided for treating NF-.kappa.B-related conditions. In particular, the invention provides a stimulus-inducible IKK signalsome, and components and variants thereof. An IKK signalsome or component thereof may be used, for example, to identify antibodies and other modulating agents that inhibit or activate signal transduction via the NF-.kappa.B cascade. IKK signalsome, components thereof and/or modulating agents may also be used for the treatment of diseases associated with NF-.kappa.B activation.

4 Claims, 30 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 28

---------- KWIC ---------

Brief Summary Text - BSTX:

The present invention relates generally to compositions and methods useful for the study of cascades leading to the activation of nuclear factor .kappa.B (NF-.kappa.B) and for treating diseases associated with such pathways. The invention is more particularly related to a stimulus-inducible I.kappa.B **kinase (IKK**) signalsome, component I.kappa.B **kinases** and variants of such **kinases**. The present invention is also related to the use of a stimulus-inducible **IKK** signalsome or I.kappa.B **kinase** to identify antibodies and other agents that inhibit or activate signal transduction via the NF-.kappa.B pathway.

Brief Summary Text - BSTX:

In further aspects, the present invention provides methods for modulating NF-.kappa.B activity in a patient, comprising administering to a patient an agent that modulates I.kappa.B **kinase** activity in combination with a pharmaceutically acceptable carrier. Methods are also provided for treating a patient afflicted with a disorder associated with the activation of **IKK** signalsome, comprising administering to a patient a therapeutically effective amount of an agent that modulates I.kappa.B **kinase** activity in combination with a pharmaceutically acceptable carrier.

Brief Summary Text - BSTX:

In a further aspect, the present invention provides a method for identifying an upstream **kinase** in the NF-.kappa.B signal transduction cascade, comprising evaluating the ability of a candidate upstream **kinase** to phosphorylate an **IKK** signalsome, a component thereof or a variant of such a component.

Brief Summary Text - BSTX:

In yet another aspect, a method is provided for preparing an **IKK** signalsome from a biological sample, comprising: (a) separating a biological sample into two or more fractions; and (b) monitoring I.kappa.B **kinase** activity in the fractions.

Drawing Description Text - DRTX:

FIGS. 8A-8C are autoradiograms depicting the results of immunoblot analyses. In FIG. 8A, the upper panel presents a time course for the induction of signalsome activity. Anti MKP-1 immune precipitates from extracts of HeLa S3 cells stimulated with TNF.alpha. (20 ng/ml) for the indicated times, were assayed for **IKK** signalsome activity by standard immune complex **kinase** assays. 4 .mu.g of either GST I.kappa.B.alpha. 1-54 WT (wildtype) or the GST I.kappa.B.alpha. 1-54 S32/36 to T mutant (S>T) were used as the substrates. In the lower panel, HeLa cell extracts prepared as described in the upper panel were examined by western blot analysis for I.kappa.B.alpha. degradation. i.kappa.B.alpha. supershifting phosphorylation can be seen after 3 and 5

minutes of stimulation followed by the disappearance of I.kappa.B.alpha..

Drawing Description Text - DRTX:

FIG. 10 is an autoradiogram showing the results of a western blot analysis of the level of ubiquitin within a stimulus-inducible IkB **kinase** complex. Lane I shows the detection of 100 ng ubiquitin, Lane 2 shows 10 ng ubiquitin and Lane 3 shows 3.4 .mu.g of **IKK** signalsome purified through the phenyl superose step (sufficient quantities for 10 **kinase** reactions). The position of ubiquitin is shown by the arrow on the left.

Drawing Description Text - DRTX:

FIG. 11A illustrates a procedure for purification of the **IKK** signalsome. A whole cell extract was prepared from TNF.alpha.-stimulated (20 ng/ml, 7 minute induction) HeLa S3 cells (1.2 g total protein). The **IKK** signalsome was then immunoprecipitated from the extract using anti-MKP-1 antibodies, washed with buffer containing 3.5 M urea and eluted overnight at 4.degree. C. in the presence of excess MKP-1 specific peptide. Eluted **IKK** signalsome was then fractionated on a Mono Q column, I.kappa.B **kinase** active fractions were pooled, concentrated and subjected to preparative SDS-PAGE. Individual protein bands were excised and submitted for peptide sequencing.

Drawing Description Text - DRTX:

FIG. 13A illustrates the amino acid sequence of **IKK-1 and IKK-2**. Symbols: arrows, boundaries of the **kinase** domain; underlined, peptide sequences identified by nanoelectrospray mass spectrometry; asterisks, indicates leucines comprising the leucine zipper motif; bold face, indicate amino acid identities conserved between **IKK-1 and IKK-2**; highlighted box, Helix-loop-helix domain; dashes, a gap inserted to optimize alignment.

Drawing Description Text - DRTX:

FIG. 13B is an autoradiogram depicting the results of Northern blot analysis of **IKK-2** mRNA in adult **human** tissue. The source of the tissue is indicated at the top. Probes spanning the coding region of **human IKK-2** and .beta.-actin cDNA were used and are indicated to the left. Molecular weight standards are indicated to the right.

Drawing Description Text - DRTX:

FIG. 14A is an autoradiogram depicting the results of **kinase** assays using **IKK-1 and IKK-2**. **IKK-1 and IKK-2** were immunoprecipitated from rabbit reticulocyte lysates phosphorylate I.kappa.B.alpha. and I.kappa.B.beta.. Either HA-tagged **IKK-1** (lane 1) or Flag-tagged **IKK-2** (lane 2) were translated in rabbit reticulocyte lysates, immunoprecipitated, and examined for their ability to

phosphorylate GST I.kappa.B.alpha. 1-54 WT and GST I.kappa.B.beta. 1-44 as indicated by an arrow to the left. **IKK-1** (lane 1) undergoes significant autophosphorylation in contrast to **IKK-2** (lane 2) which is identified only with longer exposure times.

Drawing Description Text - DRTX:

FIGS. 14B and 14C are micrographs illustrating the results of assays to evaluate the ability of **kinase**-inactive mutants of **IKK-1 and IKK-2** to inhibit RelA translocation in TNF.alpha.-stimulated HeLa cells. HeLa cells were transiently transfected with either HA-tagged **IKK-1** K44 to M mutant (14B) or Flag-tagged **IKK-2** K44 to M mutant (14C) expression vectors. 36 hours post-transfection cells were either not stimulated (Unstim) or TNF.alpha.-stimulated (20 ng/ml) for 30 min (TNF.alpha.), as indicated to the right of the figure. Cells were then subjected to immunofluorescence staining using anti-HA of anti-Flag antibodies to visualize expression of **IKK-1** K44 to M or **IKK-2** K44 to M, respectively. Stimulus-dependent translocation of Rel A was monitored using anti-Rel A antibodies. Antibodies used are indicated to the top of the figure. **IKK** mutant transfected is indicated to the left of the figure.

Detailed Description Text - DETX:

As noted above, the present invention is generally directed to compositions and methods for modulating (i.e., stimulating or inhibiting) signal transduction leading to NF-.kappa.B activation. In particular, the present invention is directed to compositions comprising an I.kappa.B **kinase (IKK**) signalsome (also referred to herein as a "stimulus-inducible I.kappa.B **kinase** complex" or "I.kappa.B **kinase** complex") that is capable of stimulus-dependent phosphorylation of I.kappa.B.alpha. and I.kappa.B.beta. on the two N-terminal serine residues critical for the subsequent ubiquitination and degradation in vivo. Such stimulus-dependent phosphorylation may be achieved without the addition of exogenous cofactors. In particular, an **IKK** signalsome specifically phosphorylates I.kappa.B.alpha. (SEQ ID NO:1) at residues S32 and S36 and phosphorylates I.kappa.B.beta. (SEQ ID NO:2) at residues S19 and S23. The present invention also encompasses compositions that contain one or more components of such an **IKK** signalsome, or variants of such components. Preferred components, referred to herein as "**IKK** signalsome **kinases**" "I.kappa.B **kinases" or IKKs) are kinases** that, when incorporated into an **IKK** signalsome, are capable of phosphorylating I.kappa.B.alpha. at S32 and S36. Particularly preferred components are **IKK-1** (SEQ ID NO:10) and **IKK-2** (SEQ ID NO:9).

Detailed Description Text - DETX:

An **IKK** signalsome and/or I.kappa.B **kinase** may generally be used for phosphorylating a substrate (i.e., an I.kappa.B, such as I.kappa.B.alpha., or a portion or variant thereof that can be phosphorylated at those residues that are phosphorylated in vivo) and for identifying modulators of I.kappa.B **kinase** activity. Such modulators and methods employing them for modulating

I.kappa.B.alpha. **kinase** activity, in vivo and/or in vitro, are also encompassed by the present invention. In general, compositions that inhibit I.kappa.B **kinase** activity may inhibit I.kappa.B phosphorylation, or may inhibit the activation of an I.kappa.B **kinase** and/or **IKK** signalsome.

Detailed Description Text - DETX:

An **IKK** signalsome has several distinctive properties. Such a complex is stable (i.e., its components remain associated following purification as described herein) and has a high-molecular weight (about 500-700 kD, as determined by gel filtration chromatography). As shown in FIGS. 3(A and B) and 4(A and B), I.kappa.B **kinase** activity of an **IKK** signalsome is "stimulus-inducible" in that it is stimulated by TNF.alpha. (i.e., treatment of cells with TNF.alpha. results in increased I.kappa.B **kinase** activity and I.kappa.B degradation) and/or by one or more other inducers of NF-.kappa.B, such as IL-1, LPS, TPA, UV irradiation, antigens, viral proteins and stress-inducing agents. The kinetics of stimulation by TNF.alpha. correspond to those found in vivo. I.kappa.B **kinase** activity of an **IKK** signalsome is also specific for S32 and S36 of I.kappa.B.alpha.. As shown in FIGS. 5(A and B) and 6(A and B), an **IKK** signalsome is capable of phosphorylating a polypeptide having the N-terminal sequence of I.kappa.B.alpha. (GST-I.kappa.B.alpha.1-54; SEQ ID NO:3), but such phosphorylation cannot be detected in an I.kappa.B.alpha. derivative containing threonine substitutions at positions 32 and 36. In addition, I.kappa.B **kinase** activity is strongly inhibited by a doubly phosphorylated I.kappa.B.alpha. peptide (i.e., phosphorylated at S32 and S36), but not by an unrelated c-fos phosphopeptide that contains a single phosphothreonine. A further characteristic of an **IKK** signalsome is its ability to phosphorylate a substrate in vitro in a standard **kinase** buffer, without the addition of exogenous cofactors. Free ubiquitin is not detectable in preparations of **IKK** signalsome (see FIG. 10), even at very long exposures. Accordingly an **IKK** signalsome differs from the ubiquitin-dependent I.kappa.B.alpha. **kinase** activity described by Chen et al., Cell 84:853-62, 1996.

Detailed Description Text - DETX:

An **IKK** signalsome may be immunoprecipitated by antibodies raised against MKP-1 (MAP **kinase** phosphatase-1; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif. #SC-1102), and its activity detected using an in vitro I.kappa.B.alpha. **kinase** assay. However, as discussed further below, MKP-1 does not appear to be a component of I.kappa.B **kinase** complex. The substrate specificity of the immunoprecipitated **IKK** signalsome is maintained (i.e., there is strong phosphorylation of wildtype GST-I.kappa.B.alpha. 1-54 (SEQ ID NO:3) and GST-I.kappa.B.beta. 1-44 (SEQ ID NO:4), and substantially no detectable phosphorylation of GST-I.kappa.B.alpha.. 1-54 in which serines 32 and 36 are replaced by threonines (GST- I.kappa.B.alpha. S32/36 to T; SEQ ID NO:5) or GST-I.kappa.B.beta. 1-44 in which serines 19 and 23 are replaced by alanines (GST-I.kappa.B.beta. 1-44 S19/23 to A; SEQ ID NO:6)).

Detailed Description Text - DETX:

An **IKK** signalsome may be isolated from **human** or other cells, and from any of a variety of tissues and/or cell types. For example, using standard protocols, cytoplasmic and/or nuclear/membrane extracts may be prepared from HeLa S3 cells following seven minutes induction with 30 ng/mL TNF.alpha.. The extracts may then be subjected to a series of chromatographic steps that includes Q Sepharose, gel filtration (HiLoad 16/60 Superdex 200), Mono Q, Phenyl Superose, gel filtration (Superdex 200 10/30) and Mono Q. This representative purification procedure is illustrated in FIG. 2, and results in highly enriched **IKK** signalsome (compare, for example, FIGS. 5A and 6A).

Detailed Description Text - DETX:

Throughout the fractionation, an in vitro **kinase** assay may be used to monitor the I.kappa.B **kinase** activity of the **IKK** signalsome. In such an assay, the ability of a fraction to phosphorylate an appropriate substrate (such as I.kappa.B.alpha. (SEQ ID NO:1) or a derivative or variant thereof) is evaluated by any of a variety of means that will be apparent to those of ordinary skill in the art. For example, a substrate may be combined with a chromatographic fraction in a protein **kinase** buffer containing .sup.32 P .gamma.-ATP, phosphatase inhibitors and protease inhibitors. The mixture may be incubated for 30 minutes at 30.degree. C. The reaction may then be stopped by the addition of SDS sample buffer and analyzed using SDS-PAGE with subsequent autoradiography. Suitable substrates include full length I.kappa.B.alpha. (SEQ ID NO: 1), polypeptides comprising the N-terminal 54 amino acids of I.kappa.B.alpha., full length I.kappa.B.beta. (SEQ ID NO:2) and polypeptides comprising the N-terminal 44 amino acids of I.kappa.B.beta.. Any of these substrates may be used with or without an N-terminal tag. One suitable substrate is a protein containing residues 1-54 of I.kappa.B.alpha. and an N-terminal GST tag (referred to herein as GST-I.kappa.B.alpha. 1-54; SEQ ID NO:3). To evaluate the specificity of an I.kappa.B **kinase** complex, I.kappa.B.alpha. mutants containing threonine or alanine residues at positions 32 and 36, and/or other modifications, may be employed.

Detailed Description Text - DETX:

Alternatively, partial sequences of the components may be obtained using standard biochemical purification and microsequencing techniques. For example, purified complex as described above may be run on an SDS-PAGE gel and individual bands may be isolated and subjected to protein microsequencing. DNA sequences encoding components may then be prepared by amplification from a suitable **human** cDNA library, using polymerase chain reaction (PCR) and methods well known to those of ordinary skill in the art. For example, an adapter-ligated cDNA library prepared from a cell line or tissue that expresses **IKK** signalsome (such as HeLa or Jurkat cells) may be screened using a degenerate 5' specific forward primer and an adapter-specific primer. Degenerate oligonucleotides may also be used to screen a cDNA library, using methods well known to those of ordinary skill in the art. In addition, known proteins may be identified via Western blot analysis using specific antibodies.

Detailed Description Text - DETX:

Components of an **IKK** signalsome may also be identified by performing any of a variety of protein-protein interaction assays known to those of ordinary skill in the art. For example, a known component can be used as "bait" in standard two-hybrid screens to identify other regulatory molecules, which may include **IKK-1, IKK-2,** NF.kappa.B1, RelA, I.kappa.B.beta. and/or p70 S6 **kinase** (Kieran et al., Cell 62:1007-1018, 1990; Nolan et al., Cell 64:961-69, 1991; Thompson et al., Cell 80:573-82, 1995; Grove et al., Mol. Cell. Biol. 11:5541-50, 1991).

Detailed Description Text - DETX:

Particularly preferred components of **IKK** signalsome are I.kappa.B **kinases**. An I.kappa.B **kinase** may be identified based upon its ability to phosphorylate one or more I.kappa.B proteins, which may be readily determined using the representative **kinase** assays described herein. In general, an I.kappa.B **kinase** is incorporated into an **IKK** signalsome, as described herein, prior to performing such assays, since an I.kappa.B **kinase** that is not complex-associated may not display the same phosphorylation activity as complex-associated I.kappa.B **kinase**. As noted above, an I.kappa.B **kinase** within an **IKK** signalsome specifically phosphorylates I.kappa.B.alpha. at residues S32 and S36, and phosphorylates I.kappa.B.beta. at residues 19 and 23, in response to specific stimuli.

Detailed Description Text - DETX:

As noted above, **IKK-1 and IKK-2** are particularly preferred I.kappa.B **kinases**. **IKK-1 and IKK-2** may be prepared by pooling the fractions from the Mono Q column containing peak I.kappa.B **kinase** activity and subjecting the pooled fractions to preparative SDS gel electrophoresis. The intensity of two prominent protein bands of .about.85 and .about.87 kDa (indicated by silver stain in FIG. 11B as **IKK-1 and IKK-2** respectively) correlates with the profile of I.kappa.B **kinase** activity. The .about.85 kDa band, corresponding to **IKK-1,** has been identified, within the context of the present invention, as CHUK (conserved helix-loop-helix ubiquitous **kinase**; see Connely and Marcu, Cell. Mol. Biol. Res. 41:537-49,1995). The .about.87 kDa band contains **IKK-2**.

Detailed Description Text - DETX:

Sequence analysis reveals that **IKK-1 and IKK-2** are related protein serine **kinases** (51% identity) containing protein interaction motifs (FIG. 13A). Both **IKK-1 and IKK-2** contain the **kinase** domain at the N-terminus, and a leucine zipper motif and a helix-loop-helix motif in their C-terminal regions. Northern analysis indicates that mRNAs encoding **IKK-2** are widely distributed in **human** tissues, with transcript sizes of .about.4.5 kb and 6 kb (FIG. 13B). The sequences of **IKK-1 and IKK-2** are also provided as SEQ ID NOs: 7 and 8, respectively.

Detailed Description Text - DETX:

It has been found, within the context of the present invention, that rabbit reticulocyte lysate immunoprecipitates that contain **IKK-1 or IKK-2** phosphorylate I.kappa.B.alpha. and I.kappa.B.beta. with the correct substrate specificity (see FIG. 14A). Altered versions of these **kinases** interfere with translocation of RelA to the nucleus of TNF.alpha.-stimulated HeLa cells. Accordingly, **IKK-1 and IKK-2** appear to control a significant early step of NF.kappa.B activation.

Detailed Description Text - DETX:

Other components of an **IKK** signalsome are also contemplated by the present invention. Such components may include, but are not limited to, upstream **kinases** such as MEKK-1 (Lee et al., Cell 88,:213-22, 1997; Hirano et al., J Biol. Chem. 271:13234-38, 1996) or NIK (Malinin et al., Nature 385:540-44, 1997); adapter proteins that mediate an **IKK**-1:IKK-2 interaction; a component that crossreacts with anti-MKP-1; an inducible RelA **kinase**; and/or the E3 ubiquitin ligase that transfers multiubiquitin chains to phosphorylated I.kappa.B (Hershko and Ciechanover, Annu. Rev. Biochem. 61:761-807, 1992).

Detailed Description Text - DETX:

In one aspect of the present invention, an **IKK** signalsome and/or one or more components thereof may be used to identify modulating agents, which may be antibodies (e.g., monoclonal), polynucleotides or other drugs, that inhibit or stimulate signal transduction via the NF-.kappa.B cascade. Modulation includes the suppression or enhancement of NF-.kappa.B activity. Modulation may also include suppression or enhancement of I.kappa.B phosphorylation or the stimulation or inhibition of the ability of activated (i.e., phosphorylated) **IKK** signalsome to phosphorylate a substrate. Compositions that inhibit NF-.kappa.B activity by inhibiting I.kappa.B phosphorylation may include one or more agents that inhibit or block I.kappa.B.alpha. **kinase** activity, such as an antibody that neutralizes **IKK** signalsome, a competing peptide that represents the substrate binding domain of I.kappa.B **kinase** or a phosphorylation motif of I.kappa.B, an antisense polynucleotide or ribozyme that interferes with transcription and/or translation of I.kappa.B **kinase,** a molecule that inactivates **IKK** signalsome by binding to the complex, a molecule that binds to I.kappa.B and prevents phosphorylation by **IKK** signalsome or a molecule that prevents transfer of phosphate groups from the **kinase** to the substrate. Within certain embodiments, a modulating agent inhibits or enhances the expression or activity of **IKK-1** and/or **IKK-2**.

Detailed Description Text - DETX:

In general, modulating agents may be identified by combining a test compound with an **IKK** signalsome, I.kappa.B **kinase** or a polynucleotide encoding an I.kappa.B **kinase** in vitro or in vivo, and evaluating the effect of the test

compound on the I.kappa.B **kinase** activity using, for example, a representative assay described herein. An increase or decrease in **kinase** activity can be measured by adding a radioactive compound, such as .sup.32 P-ATP and observing radioactive incorporation into a suitable substrate for **IKK** signalsome, thereby determining whether the compound inhibits or stimulates **kinase** activity. Briefly, a candidate agent may be included in a reaction mixture containing compounds necessary for the **kinase** reaction (as described herein) and I.kappa.B substrate, along with **IKK** signalsome, I.kappa.B **kinase** or a polynucleotide encoding an I.kappa.B **kinase**. In general, a suitable amount of antibody or other agent for use in such an assay ranges from about 0.01 .mu.M to about 10 .mu.M. The effect of the agent on I.kappa.B **kinase** activity may then be evaluated by quantitating the incorporation of [.sup.32 P]phosphate into an I.kappa.B such as I.kappa.B.alpha. (or a derivative or variant thereof), and comparing the level of incorporation with that achieved using I.kappa.B **kinase** without the addition of a candidate agent. Alternatively, the effect of a candidate modulating agent on transcription of an I.kappa.B **kinase** may be measured, for example, by Northern blot analysis or a promoter/reporter-based whole cell assay.

Detailed Description Text - DETX:

Alternatively, for assays in which the test compound is combined with an **IKK** signalsome, the effect on a different **IKK** signalsome activity may be assayed. For example, an **IKK** signalsome also displays p65 **kinase** activity and **IKK** phosphatase activity. Assays to evaluate the effect of a test compound on such activities may be performed using well known techniques. For example, assays for p65 **kinase** activity may generally be performed as described by Zhong et al., Cell 89:413-24, 1997. For phosphatase activity, an assay may generally be performed as described by Sullivan et al., J. Biomolecular Screening 2:19-24, 1997, using a recombinant phosphorylated I.kappa.B **kinase** as a substrate.

Detailed Description Text - DETX:

In another aspect of the present invention, **IKK** signalsome or I.kappa.B **kinase** may be used for phosphorylating an I.kappa.B such as I.kappa.B.alpha. (or a derivative or variant thereof) so as to render it a target for ubiquitination and subsequent degradation. I.kappa.B may be phosphorylated in vitro by incubating **IKK** signalsome or I.kappa.B **kinase** with I.kappa.B in a suitable buffer for 30 minutes at 30.degree. C. In general, about 0.01 .mu.g to about 9 .mu.g of I.kappa.B **kinase** complex is sufficient to phosphorylate from about 0.5 .mu.g to about 2 .mu.g of I.kappa.B. Phosphorylated substrate may then be purified by binding to GSH-sepharose and washing. The extent of substrate phosphorylation may generally be monitored by adding [.gamma.-.sup.32 P]ATP to a test aliquot, and evaluating the level of substrate phosphorylation as described herein.

Detailed Description Text - DETX:

An **IKK** signalsome, component thereof, modulating agent and/or polynucleotide

encoding a component and/or modulating agent may also be used to modulate NF-.kappa.B activity in a patient. Such modulation may occur by any of a variety of mechanisms including, but not limited to, direct inhibition or enhancement of I.kappa.B phosphorylation using a component or modulating agent; or inhibiting upstream activators, such as NIK or MEK, with **IKK** signalsome or a component thereof. As used herein, a "patient" may be any mammal, including a **human,** and may be afflicted with a disease associated with I.kappa.B **kinase** activation and the NF-.kappa.B cascade, or may be free of detectable disease. Accordingly, the treatment may be of an existing disease or may be prophylactic. Diseases associated with the NF-.kappa.B cascade include inflammatory diseases, neurodegenerative diseases, autoimmune diseases, cancer and viral infection.

Detailed Description Text - DETX:

Treatment may include administration of an **IKK** signalsome, a component thereof and/or an agent which modulates I.kappa.B **kinase** activity. For administration to a patient, one or more such compounds are generally formulated as a pharmaceutical composition. A pharmaceutical composition may be a sterile aqueous or non-aqueous solution, suspension or emulsion, which additionally comprises a physiologically acceptable carrier (i.e., a non-toxic material that does not interfere with the activity of the active ingredient). Any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of the present invention. Representative carriers include physiological saline solutions, gelatin, water, alcohols, natural or synthetic oils, saccharide solutions, glycols, injectable organic esters such as ethyl oleate or a combination of such materials. Optionally, a pharmaceutical composition may additionally contain preservatives and/or other additives such as, for example, antimicrobial agents, anti-oxidants, chelating agents and/or inert gases, and/or other active ingredients.

Detailed Description Text - DETX:

In another aspect, the present invention provides methods for detecting the level of stimulus-inducible I.kappa.B **kinase** activity in a sample. The level of I.kappa.B **kinase** activity may generally be determined via an immunokinase assay, in which **IKK** signalsome is first immunoprecipitated with an antibody that binds to the complex. The immunoprecipitated material is then subjected to a **kinase** assay as described herein. Substrate specificity may be further evaluated as described herein to distinguish the activity of a stimulus-inducible I.kappa.B **kinase** complex from other **kinase** activities.

Detailed Description Text - DETX:

The present invention also provides methods for detecting the level of **IKK** signalsome, or a component thereof, in a sample. The amount of **IKK** signalsome, I.kappa.B **kinase** or nucleic acid encoding I.kappa.B **kinase,** may generally be determined using a reagent that binds to I.kappa.B **kinase,** or to DNA or RNA encoding a component thereof. To detect nucleic acid encoding a component,

standard hybridization and/or PCR techniques may be employed using a nucleic acid probe or a PCR primer. Suitable probes and primers may be designed by those of ordinary skill in the art based on the component sequence. To detect **IKK** signalsome or a component thereof, the reagent is typically an antibody, which may be prepared as described below.

Detailed Description Text - DETX:

Antibodies encompassed by the present invention may be polyclonal or monoclonal, and may bind to **IKK** signalsome and/or one or more components thereof (e.g., **IKK-1** and/or **IKK-2**). Preferred antibodies are those antibodies that inhibit or block I.kappa.B **kinase** activity in vivo and within an in vitro assay, as described above. Other preferred antibodies are those that bind to one or more I.kappa.B proteins. As noted above, antibodies and other agents having a desired effect on I.kappa.B **kinase** activity may be administered to a patient (either prophylactically or for treatment of an existing disease) to modulate the phosphorylation of an I.kappa.B, such as I.kappa.B.alpha., in vivo.

Detailed Description Text - DETX:

In a related aspect of the present invention, kits for detecting the level of **IKK** signalsome, I.kappa.B **kinase,** nucleic acid encoding I.kappa.B **kinase** and/or I.kappa.B **kinase** activity in a sample are provided. Any of a variety of samples may be used in such assays, including eukaryotic cells, bacteria, viruses, extracts prepared from such organisms and fluids found within living organisms. In general, the kits of the present invention comprise one or more containers enclosing elements, such as reagents or buffers, to be used in the assay.

Detailed Description Text - DETX:

A kit for detecting the level of **IKK** signalsome, I.kappa.B **kinase** or nucleic acid encoding I.kappa.B **kinase** typically contains a reagent that binds to the compound of interest. To detect nucleic acid encoding I.kappa.B **kinase,** the reagent may be a nucleic acid probe or a PCR primer. To detect **IKK** signalsome or I.kappa.B **kinase,** the reagent is typically an antibody. Such kits also contain a reporter group suitable for direct or indirect detection of the reagent (i.e., the reporter group may be covalently bound to the reagent or may be bound to a second molecule, such as Protein A, Protein G, immunoglobulin or lectin, which is itself capable of binding to the reagent). Suitable reporter groups include, but are not limited to, enzymes (e.g., horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. Such reporter groups may be used to directly or indirectly detect binding of the reagent to a sample component using standard methods known to those of ordinary skill in the art.

Detailed Description Text - DETX:

In yet another aspect, **IKK** signalsome may be used to identify one or more native upstream **kinases** (i.e., **kinases** that phosphorylate and activate **IKK** signalsome in vivo) or other regulatory molecules that affect I.kappa.B **kinase** activity (such as phosphatases or molecules involved in ubiquitination), using methods well known to those of ordinary skill in the art. For example, **IKK** signalsome components may be used in a yeast two-hybrid system to identify proteins that interact with such components. Alternatively, an expression library may be screened for cDNAs that phosphorylate **IKK** signalsome or a component thereof.

Detailed Description Text - DETX:

This example illustrates the recruitment of NF.kappa.B into a protein complex (the **IKK** signalsome) containing I.kappa.B **kinase** and other signaling proteins.

Detailed Description Text - DETX:

As shown in FIG. 1A, I.kappa.B.alpha. in cell extracts from unstimulated cells eluted with an apparent molecular weight of .about.300 kDa (lanes 5-7), consistent with the chromatographic properties of the inactive NF.kappa.B-I.kappa.B complex (Baeuerle and Baltimore, Genes Dev. 3:1689-98, 1989). In contrast, phosphorylated I.kappa.B.alpha. (from cells stimulated for periods too short to permit complete degradation of the protein) migrated at .about.600 kDa on the same chromatography columns (lanes 2, 3). This difference in migration was specific for I.kappa.B, since analysis of the same fractions indicated that the Jun N-terminal **kinases** JNK1 and JNK2 migrated with low apparent molecular weight and showed no difference in chromatographic behavior between stimulated and unstimulated cells. Stimulation-dependent recruitment of I.kappa.B into this larger protein complex corresponded with the appearance of phosphorylated I.kappa.B, suggesting that the complex contained the specific I.kappa.B **kinases** that mediate I.kappa.B phosphorylation. These results demonstrate that that NF.kappa.B activation involves recruitment into a protein complex (the **IKK** signalsome) containing I.kappa.B **kinase** and other signaling proteins.

Detailed Description Text - DETX:

NF.kappa.B activation is known to occur under conditions that also stimulate MAP **kinase** pathways (Lee et al., Cell 88:213-22, 1997; Hirano, et al., J. Biol. Chem. 271:13234-38, 1996). Accordingly, further experiments were performed to detect proteins associated with MAP **kinase** and phosphatase cascades at various stages of purification of the **IKK** signalsome. In addition to RelA and I.kappa.B.beta., MEKK-1 and two tyrosine-phosphorylated proteins of .about.55 and .about.40 kDa copurified with I.kappa.B **kinase** activity (FIG. 1C). Antibodies to Rel A and I.kappa.B.beta. were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.), and antibodies to MEKK-1 were obtained from Upstate Biotechnology (Lake Placid, N.Y.). Other signaling components, including PKC.zeta., PP1 and PP2A, were detected in the same

fractions as the I.kappa.B **kinase** in early chromatographic steps but did not copurify at later chromatographic steps (data not shown). Most interestingly, an unidentified protein of .about.50 kDa, detected by its crossreaction with an antibody to MKP-1, copurified with I.kappa.B **kinase** through several purification steps (FIG. 1C). This protein is unlikely to be MKP-1 itself, since the molecular weight of authentic MKP-1 is 38 kDa.

Detailed Description Text - DETX:

Of a large panel of antibodies tested, one of three anti-MKP-1 antibodies efficiently co-immunoprecipitated an inducible I.kappa.B **kinase** activity from HeLa cells as well as primary **human** umbilical vein endothelial cells (HUVEC). The co-immunoprecipitated **kinase (IKK** signalsome **kinase**) was inactive in unstimulated HeLa cells, but was rapidly activated within minutes of TNF.alpha. stimulation (FIG. 8A, top panel). The **IKK** signalsome **kinase** did not phosphorylate a mutant GST-I.kappa.B.alpha. protein in which serine residues 32 and 36 had been mutated to threonine (FIG. 8A top panel, even-numbered lanes). Activation of the signalsome **kinase** was maximal at 5 minutes and declined thereafter, a time course consistent with the time course of I.kappa.B.alpha. phosphorylation and degradation under the same conditions (FIG. 8A, bottom panel). As expected, the signalsome I.kappa.B **kinase** was also activated by stimulation of cells with IL-1 or PMA (FIG. 8B, lanes 1-4); moreover, its activity was inhibited in cells treated with TPCK, a known inhibitor of NF.kappa.B activation (FIG. 8B, lane 7). Additionally, the **IKK** signalsome **kinase** specifically phosphorylated full-length wild-type I.kappa.B.alpha., but not a mutant I.kappa.B.alpha. bearing the serine 32, 36 to alanine mutations, in the context of a physiological RelA-I.kappa.B.alpha. complex (FIG. 8C, lanes 3, 4). Together these results indicate that the anti-MKP-1 antibody co-immunoprecipitated the **IKK** signalsome. The signalsome-associated I.kappa.B **kinase** met all the criteria expected of the authentic I.kappa.B **kinase** and had no detectable I.kappa.B.alpha. C-terminal **kinase** activity.

Detailed Description Text - DETX:

The specificity of the **IKK** signalsome **kinase** was further established by kinetic analysis and by examining its activity on various peptides and recombinant protein substrates (FIG. 9A). For these studies, synthetic peptides (Alpha Diagnostics International, San Antonio, TX) were prepared with the following sequences:

Detailed Description Text - DETX:

The **kinase** displayed normal Michaelis-Menten kinetics, suggesting that it was not a mixture of diverse unrelated **kinases**. The **kinase** was capable of phosphorylating an I.kappa.B.alpha. (21-41) peptide (FIGS. 9A and 9B)) as well as two different I.kappa.B.alpha. (21-41) peptides, each bearing a free serine at either position 32 or 36 and phosphoserine at the other position (FIGS. 9A and 9B, lanes 2, 3). As expected, a peptide with phosphoserines at both

positions was not phosphorylated at all (FIG. 9B, top), indicating that there was no significant turnover of the phosphates under our reaction conditions. These experiments indicated that both serines 32 and 36 were phosphoacceptor sites for the **IKK** signalsome **kinase,** thus distinguishing it from other **kinases** such as pp90Rsk which phosphorylates I.kappa.B.alpha. only at serine 32 (Schouten, et al., EMBOJ 16:3133-44, 1997).

Detailed Description Text - DETX:

Although the **IKK** signalsome **kinase** efficiently phosphorylated I.kappa.B peptides, it did not phosphorylate the c-Fos phosphopeptide containing a free serine and a free threonine (FIG. 9B, top), two c-Jun peptides containing serine 63 and 73, respectively, (FIG. 9A, top panel, lanes 4, 5), or an MKP-1 peptide containing four serines and three threonines (FIG. 9A, lane 6). The latter peptides were substrates for JNK2 (FIG. 9A, bottom panel, lanes 4-6). An I.kappa.Boc (21-41) peptide in which serines 32 and 36 were replaced by threonines was phosphorylated by the signalsome at least 10-fold less well than the wild-type serine-containing peptide, consistent with the slower phosphorylation and degradation kinetics of I.kappa.B.alpha. (S32/36 to T) in cells (DiDonato et al., Mol. Cell. Biol. 16:1295-1304, 1996), and the preference of the **kinase** for serine over threonine at positions 32, 36 in both full-length I.kappa.B.alpha. and GST-I.kappa.B.alpha. (1-54) (FIGS. 8A and C). In addition, the **kinase** phosphorylated GST-I.kappa.B.beta. (1-54), albeit with lower affinity. In most experiments, I.kappa.B **kinase** activity was also associated with strong RelA **kinase** activity (FIG. 8C, lanes 3, 4), but no activity was observed towards several other substrates including myelin basic protein (MBP), GST-ATF2 (1-112), GST-cJun (1-79), GST-ERK3, GST-Elk (307-428), GST-p38, and a GST fusion protein containing the C-terminal region of I.kappa.B.alpha. (242-314).

Detailed Description Text - DETX:

The specificity of the **IKK** signalsome **kinase** was further emphasized by its susceptibility to product inhibition (FIG. 9B, bottom). The **kinase** was strongly inhibited by a doubly-phosphorylated I.kappa.B.alpha. peptide bearing phosphoserines at both positions 32 and 36, but not by the unrelated c-Fos phosphopeptide that contained a single phosphothreonine. The singly-phosphorylated and the unphosphorylated I.kappa.B.alpha. peptides were less effective inhibitors.

Detailed Description Text - DETX:

This example illustrates the absence of detectable free ubiquitin with a **IKK** signalsome prepared as in Example 3. Standard western blot procedures were performed (Amersham Life Science protocol, Arlington Heights, Ill.). 100 ng ubiquitin, 10 ng ubiquitin and 20 ul purified IkB.alpha. **kinase** complex was subjected to 16% Tricine SDS-PAGE (Novex, San Diego, Calif.), transferred to Hybond ECL Nitrocellulose membrane (Amersham Life Science, Arlington Heights, Ill.), and probed with antibodies directed against ubiquitin (MAB 1510;

Chemicon, Temecula, Calif.). The results are shown in FIG. 10. Free ubiquitin could not be detected in the purified IkB.alpha. **kinase** preparation (even at very long exposures). The complexes described herein do not require addition of endogenous ubiquitin to detect IkB.alpha. **kinase** activity, nor is free ubiquitin a component in the purified IkB.alpha. **kinase** preparations of the present invention.

Detailed Description Text - DETX:

This Example illustrates a two-step affinity method for purification of the **IKK** signalsome, based on its recognition by the MKP-1 antibody (depicted in FIG. 11A) and the identification of I.kappa.B **kinases**.

Detailed Description Text - DETX:

For large scale **IKK** signalsome purification, HeLa S3 cells were stimulated for 7 minutes with 20 ng/ml TNF.alpha. (R&D Systems, Minneapolis, Minn.), harvested, whole cell lysates were prepared (1.2 g total protein) and approximately 5 mg of anti-MKP-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was added and incubated at 4.degree. C. for 2 hours with gentle rotation. Subsequently, 50 ml of Protein A agarose (Calbiochem, San Diego, Calif.) was added and the mixture was incubated for an additional 2 hours. The immunoprecipitate was then sequentially washed 4.times.Pull-Down Buffer, 2.times.RIPA buffer, 2X Pull-Down Buffer, 1.times.3.5 M urea-Pull-Down Buffer and 3.times.Pull-Down Buffer. The immunoprecipitate was then made into a thick slurry by the addition of 10 ml of Pull-Down Buffer, 25 mg of the specific MKP-1 peptide to which the antibody was generated (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was added, and the mixture was incubated overnight at 4.degree. C. with gentle rotation. The eluted **IKK** signalsome was then desalted on PD 10 desalting columns (Pharmacia Biotech, Piscataway, N.J.) equilibrated with 50 mM Q buffer and chromatographed on a Mono Q column (Pharmacia Biotech, Piscataway, N.J.). Fractions containing peak IkB **kinase** activity were pooled, concentrated and subjected to preparative SDS-PAGE. The intensity of two prominent protein bands of .about.85 and .about.87 kDa (indicated by silver stain in FIG. 11B as **IKK-1 and IKK-2** respectively) correlated with the profile of I.kappa.B **kinase** activity.

Detailed Description Text - DETX:

Coomassie stained .about.85 and .about.87 kDa bands were excised, in-gel digested with trypsin (Wilm et al., Nature 3 7:466-69, 1996) and a small aliquot of the supernatant was analyzed by high mass accuracy MALDI peptide mass mapping, as described by Shevchenko et al., Proc. Natl. Acad. Sci. USA 93:14440-45, 1996. The peptide mass map from the **IKK-1** band was searched against a comprehensive protein sequence database using the program PeptideSearch developed in house at EMBL Heidelberg. Eight measured peptide masses matched calculated tryptic peptide masses from CHUK (conserved helix-loop-helix ubiquitous **kinase**; Connely and Marcu, Cell. Mol. Biol. Res. 41:537-49, 1995) within 30 ppm, unambiguously identifying the protein. The

peptide mass map of the **IKK-2** band did not result in a clear identification and therefore the sample was subjected to nanoelectrospray mass spectrometry (Wilm and Mann, Anal. Chem. 68:1-8, 1996). The peptide mixture obtained after extraction of the gel piece was micropurified on a capillary containing 50 nL of POROS R2 resin (PerSeptive Biosystems, Framingham, Mass.). After washing, the peptides were step-eluted with 0.5 .mu.L of 50% MeOH in 5% formic acid into a nanoelectrospray needle. This needle was transferred to an APIII mass spectrometer (Perkin-Elmer, Sciex, Toronto, Canada) and the sample sprayed for approximately 20 minutes. During this time, peptide ions apparent from the mass spectrum were selected and isolated in turn and fragmented in the collision chamber of the mass spectrometer. From the tandem mass spectra, short stretches of sequence were assembled into peptide sequence tags (Mann and Wilm, Anal. Chem. 66:4390-99, 1994) and searched against a protein sequence database or against dbEST using PeptideSearch.

Detailed Description Text - DETX:

Three peptides matched the **IKK-1** sequence. A1: IIDLGYAK (SEQ ID NO:17); A2: VEVALSNIK (SEQ ID NO:18); A3 SIQLDLER (SEQ ID NO:19). Three other peptides matched **human** EST sequences in dbEST: B1: ALELLPK (SEQ ID NO:20), B2: VIYTQLSK (SEQ ID NO:21), B6: LLLQAIQSFEK (SEQ ID NO:22) all match EST clone AA326115. The peptide B4 with the sequence LGTGGFGNVIR (SEQ ID NO:23) was found in clone R06591. After the full-length **IKK-2** sequence was obtained (as described below) two more peptides B3: ALDDILNLK (SEQ ID NO:24) and B5: DLKPENIVLQQGEQR (SEQ ID NO:25) were found in the sequence. Peptide A1 is present in both **IKK-1 and IKK-2** sequences. All the EST clones identified were clearly homologous to **human and mouse** CHUK, a serine/threonine **kinase** of hitherto unknown function. Once the complete coding sequence of **IKK-2** was obtained (as described below), all sequenced peptides (apart from two peptides derived from **IKK-1**) could be assigned to this protein.

Detailed Description Text - DETX:

Representative mass spectra are shown in FIGS. 12A and 12B. In FIG. 12A, peaks labeled A were matched to the tryptic peptides of **IKK-1** upon fragmentation followed by database searching with peptide sequence tags. Peaks labeled B2, B4, B6 were not found in protein databases but instead matched **human** EST sequences. One more peptide (B1) matching a **human** EST clone was observed at m/z 392.2 and is not shown in panel A. In FIG. 12B, a continuous series of C-terminal-containing fragments (Y"-ions) was used to construct a peptide sequence tag as shown by boxed letters. Search of this tag resulted in a match to the peptide LLLQALQSFEK (SEQ ID NO:22) in **human** EST clone AA326115. Two more peptides, BI (ALELLPK; SEQ ID NO:20) and B2 (VIYTQLSK; SEQ ID NO:21) were found in the sequence of the same EST clone.

Detailed Description Text - DETX:

Full-length **human IKK-1 and IKK-2** cDNAs were cloned based on the. **human** EST clones, which were obtained from Genome Systems, Inc. (St. Louis, Mo.). The

precise nucleotide sequences were determined and used to design primers to PCR clone **human IKK-2 from a human** HeLa cell cDNA library (Clontech, Inc., Palo Alto, Calif.). Several **IKK-2** cDNA clones were isolated and sequenced. Full-length **mouse IKK-1** and a partial **human IKK-1** nucleotide sequence was available in the comprehensive database, primers were designed to PCR clone the respective **human and mouse IKK-1** cDNAs. The partial **human IKK-1** coding region was used to probe a HeLa cDNA phage library (Stratagene, Inc., La Jolla, Calif.) to obtain the full-length **human IKK-1** cDNA clone using standard procedures.

Detailed Description Text - DETX:

Sequence analysis of these clones revealed that **IKK-1 and IKK-2** were related protein serine **kinases** (51% identity) containing protein interaction motifs (FIG. 13A). Both **IKK-1 and IKK-2** contain the **kinase** domain at the N-terminus, and a leucine zipper motif and a helix-loop-helix motif in their C-terminal regions (FIG. 13A). Northern analysis indicated that mRNAs encoding **IKK-2** were widely distributed in **human** tissues, with transcript sizes of .about.4.5 kb and 6 kb (FIG. 13B). The distribution of **IKK-1** (CHUK) transcripts has been reported previously (Connely et al., Cell Mol. Biol. Res. 41:537-49, 1995). **IKK-1 and IKK-2** mRNAs are constitutively expressed in Jurkat, HeLa and HUVEC cell lines, and their levels are not altered for up to 8 hours following stimulation with NF.kappa.B inducers such as TNF.alpha. (HeLa, HUVEC) or anti-CD28 plus PMA (Jurkat).

Detailed Description Text - DETX:

To further characterize the properties of **IKK-1 and IKK-2,** recombinant HA-tagged **IKK-1** and Flag-tagged **IKK-2,** either separately or alone, were in vitro transcribed and translated in wheat germ or rabbit reticulocyte lysate (Promega, Madison, Wis.). The reactions were performed exactly as described in the manufacturer's protocol. Epitope-tagged **IKK-1 and IKK-2** then immunoprecipitated with the appropriate anti-tag antibody. Immunoprecipitates containing these proteins phosphorylated I.kappa.B.alpha. and I.kappa.B.beta. with the correct substrate specificity (i.e., immunoprecipitates of **IKK-1 and IKK-2** phosphorylated both GST-I.kappa.B.alpha. (FIG. 14A, panel 3) and GST-I.kappa.B.beta. (panel 4), but did not phosphorylate the corresponding S32/36 to T mutant protein). **IKK-1** expressed in rabbit reticulocyte lysates was also capable of autophosphorylation (FIG. 14A, panel 2, lane 1), whereas a **kinase**-inactive version of **IKK-1,** in which the conserved lysine 44 had been mutated to methionine, showed no autophosphorylation. In contrast **IKK-2,** although expressed at equivalent levels in the lysates (panel 1), showed very weak autophosphorylation (panel 2, lane 2).

Detailed Description Text - DETX:

Expression of the **kinase** inactive mutants (K to M) of **IKK-1 and IKK-2** indicate that both play critical roles in NF.kappa.B activation as demonstrated by immunofluorescent studies (FIGS. 14B and 14C). For these studies, HeLa cells

were transiently transfected with either HA-tagged **IKK-1** or Flag-tagged **IKK-2**. Cells were fixed for 30 minutes with methanol. For immunofluorescence staining, the cells were incubated sequentially with primary antibody in PBS containing 10% donkey serum and 0.25% Triton X-100 for 2 hours followed by fluorescein-conjugated or Texas red-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.; used at 1:500 dilution) for 1 hour at room temperature. The coverslips were rinsed and coverslipped with Vectashield (Vector Laboratories, Burlingame, Calif.) before scoring and photographing representative fields. Primary antibodies used for immunofluorescence staining included antibodies against Rel A (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), HA tag (Babco, Berkeley, Calif.) and Flag tag (IBI-Kodak, New Haven, Conn.).

Detailed Description Text - DETX:

Kinase-inactive versions (K44 to M) of IKK-1 and **IKK-2 had no e**ffect on the subcellular localization of RelA in unstimulated HeLa cells, since RelA remained cytoplasmic both in cells expressing the epitope-tagged proteins and in the adjacent untransfected cells (FIGS. 14B and 14C, top panels). In contrast, both mutant proteins inhibited RelA nuclear translocation in TNF.alpha.-stimulated cells (FIGS. 14B and 14C, bottom panels). The inhibition mediated by the IKK-2 mut**ant w**as striking and complete (FIG. 14C: compare mutant IKK-2-exp**res**sing cells with untransfected cells in the same field), whereas that mediated by the mutant IKK-1 pro**tein,** expressed at apparently equivalent levels, was significant but incomplete (FIG. 14B). This difference in the functional activities of the two mutant kinases m**ay poin**t to distinct roles for these two kinases i**n NF.ka**ppa.B activation.

Detailed Description Text - DETX:

Both **IKK-1 and IKK-2 kinases** were active when expressed in wheat germ extracts, since they were capable of autophosphorylation, but they were inactive with respect to phosphorylation of I.kappa.B substrates. Since both autophosphorylation and substrate phosphorylation were intact in rabbit reticulocyte lysates, there appeared to be a direct correlation between the association of **IKK-1 and IKK-2** into a higher order protein complex and the presence of specific I.kappa.B **kinase** activity in **IKK-1 and IKK-2** immunoprecipitates. This higher order complex is most likely the **IKK** signalsome itself. Indeed, immunoprecipitation of rabbit reticulocyte lysates with anti-MKP-1 antibody pulls down a low level of active I.kappa.B **kinase** activity characteristic of the **IKK** signalsome.

Detailed Description Text - DETX:

It is clear that the **IKK** signalsome contains multiple protein components in addition to **IKK-1 and IKK-2** (FIG. 11B). Some of these may be upstream kinases such as MEKK-1 (Chen et al., Cell 84:853-62, 1996) or NIK (Malinin, et al., Nature 385:540-44, 1997); others may be adapter proteins that mediate the **IKK**-1:IKK-2 interaction. Indeed MEKK-1 copurifies with **IKK** signalsome activity

(FIG. 1C), and two other signalsome proteins have been functionally identified. The protein crossreactive with anti-MKP-1 is an intrinsic component of the **IKK** signalsome **kinases,** since the I.kappa.B **kinase** activity coprecipitated with this antibody is stable to washes with 2-4 M urea. Moreover, both **IKK-1** immunoprecipitates and MKP-1 immunoprecipitates containing the **IKK** signalsome (FIG. 8C) contain an inducible RelA **kinase** whose kinetics of activation parallel those of the I.kappa.B **kinase** in the same immunoprecipitates. Another strong candidate for a protein in the signalsome complex is the E3 ubiquitin ligase that transfers multiubiquitin chains to phosphorylated I.kappa.B (Hershko et al., Annu. Rev. Biochem. 61:761-807, 1992).

Detailed Description Text - DETX:

These results indicate that **IKK-1 and IKK-2** are functional **kinases** within the **IKK** signalsome, which mediate I.kappa.B phosphorylation and NF.kappa.B activation. Appropriate regulation of **IKK-1 and IKK-2** may require their assembly into a higher order protein complex, which may be a heterodimer facilitated by adapter proteins, the complete **IKK** signalsome, or some intermediate subcomplex that contains both **IKK-1 and IKK-2**.

Claims Text - CLTX:

2. An **IKK** signalsome according to claim 1 wherein the signalsome is derived from a **human** tissue or cell line.

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TITLE: IkB kinase, subunits thereof, and methods of using same

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APPL-NO: 09/ 168629

DATE FILED: October 8, 1998

PARENT-CASE:

This application is based on, and claims the benefit of, U.S. Provisional application Ser. No. 60/061,470, filed Oct. 9, 1997, the entire contents of which is herein incorporated by reference.

US-CL-CURRENT: 435/325; 435/194 ; 435/252.3 ; 435/320.1 ; 536/23.2

ABSTRACT:

The present invention provides an isolated nucleic acid molecules encoding IKB **kinase (IKK**) catalytic subunit polypeptides, which are associated with an **IKK** serine protein **kinase** that phosphorylates a protein (IKB) that inhibits the activity of the NF-KB transcription factor, vectors comprising such nucleic acid molecules and host cells containing such vectors. In addition, the invention provides nucleotide sequences that can bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful as probes or as antisense molecules. The invention also provides isolated **IKK** catalytic subunits, which can phosphorylate an IKB protein, and peptide portions of such **IKK** subunit. In addition, the invention provides anti-**IKK** antibodies, which specifically bind to an **IKK** complex or an **IKK** catalytic subunit, and **IKK**-binding fragments of such antibodies. The invention further provides methods of substantially purifying an **IKK** complex, methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** catalytic subunit with a second protein, and methods of identifying proteins that can interact with an **IKK** complex or an **IKK** catalytic subunit.

11 Claims, 5 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 5

---------- KWIC ---------

Abstract Text - ABTX:

The present invention provides an isolated nucleic acid molecules encoding IKB **kinase (IKK**) catalytic subunit polypeptides, which are associated with an **IKK** serine protein **kinase** that phosphorylates a protein (IKB) that inhibits the activity of the NF-KB transcription factor, vectors comprising such nucleic acid molecules and host cells containing such vectors. In addition, the invention provides nucleotide sequences that can bind to a nucleic acid molecule of the invention, such nucleotide sequences being useful as probes or as antisense molecules. The invention also provides isolated **IKK** catalytic subunits, which can phosphorylate an IKB protein, and peptide portions of such **IKK** subunit. In addition, the invention provides anti-**IKK** antibodies, which specifically bind to an **IKK** complex or an **IKK** catalytic subunit, and **IKK**-binding fragments of such antibodies. The invention further provides methods of substantially purifying an **IKK** complex, methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** catalytic subunit with a second protein, and methods of identifying proteins that can interact with an **IKK** complex or an **IKK** catalytic subunit.

Brief Summary Text - BSTX:

The present invention provides isolated nucleic acid molecules encoding full length **human** serine protein **kinases,** designated IKB **kinase (IKK**) subunits IKK.alpha. and IKK.beta.. The disclosed **IKK** subunits share substantial sequence homology and are activated in response to proinflammatory signals to phosphorylate proteins (IKB's) that inhibit the activity of the NF-KB transcription factor.

Brief Summary Text - BSTX:

The present invention also provides isolated full length **human IKK** subunits, which can phosphorylate an IKB protein. For example, the invention provides an IKK.alpha. polypeptide having the amino acid sequence shown as SEQ ID NO: 2, particularly the amino acid sequence comprising amino acids 1 to 31 at the N-terminus of the polypeptide of SEQ ID NO: 2. In addition, the invention provides an IKK.beta. polypeptide having the amino acid sequence shown as SEQ ID NO: 15. The invention also provides peptide portions of an **IKK** subunit, including, for example, peptide portions comprising one or more contiguous amino acids of the N-terminal amino acids shown as residues 1 to 31 in SEQ ID NO: 2. A peptide portion of an **IKK** subunit can comprise the **kinase** domain of the **IKK** subunit or can comprise a peptide useful for eliciting production of an

antibody that specifically binds to an IKB **kinase or to the IKK** subunit. Accordingly, the invention also provides anti-**IKK** antibodies that specifically bind to an **IKK** complex comprising an **IKK** subunit, particularly to the **IKK** subunit, for example, to an epitope comprising at least one of the amino acids shown as residues 1 to 31 of SEQ ID NO: 2, and also provides **IKK** subunit-binding fragments of such antibodies. In addition, the invention provides cell lines producing anti-**IKK** antibodies or **IKK**-binding fragments thereof.

Brief Summary Text - BSTX:

The invention also provides isolated IKB **kinase** complexes. As disclosed herein, an **IKK** complex can have an apparent molecular mass of about 900 kDa or about 300 kDa. An **IKK** complex is characterized, in part, in that it comprises an IKK.alpha. subunit, an IKK.beta. subunit, or both and can phosphorylate an IKB protein.

Brief Summary Text - BSTX:

The present invention further provides methods for isolating an **IKK** complex or an **IKK** subunit, as well as methods of identifying an agent that can alter the association of an **IKK** complex or an **IKK** subunit with a second protein that associates with the **IKK** in vitro or in vivo. Such a second protein can be, for example, another **IKK** subunit; an IKB protein, which is a substrate for **IKK** activity and is involved in a signal transduction pathway that results in the regulated expression of a gene; a protein that is upstream of the IKB **kinase** in a signal transduction pathway and regulates **IKK** activity; or a protein that acts as a regulatory subunit of the IKB **kinase or of an IKK** subunit and is necessary for full activation of the **IKK** complex. An agent that alters the association of an **IKK** subunit with a second protein can be, for example, a peptide, a polypeptide, a peptidomimetic or a small organic molecule. Such agents can be useful for modulating the level of phosphorylation of IKB in a cell, thereby modulating the activity of NF-KB in the cell and the expression of a gene regulated by NF-KB.

Brief Summary Text - BSTX:

The invention also provides methods of identifying proteins that can interact with an IKB **kinase,** including with an **IKK** subunit, such proteins which can be a downstream effector of the **IKK** such as a member of the IKB family of proteins or an upstream activator or a regulatory subunit of an **IKK**. Such proteins that interact with an **IKK** complex or the **IKK** subunit can be isolated, for example, by coprecipitation. with the **IKK** or by using the **IKK** subunit as a ligand, and can be involved, for example, in tissue specific regulation of NF-KB activation and consequent tissue specific gene expression.

Drawing Description Text - DRTX:

FIG. 1 shows a nucleotide sequence (SEQ ID NO: 1; lower case letter) and deduced amino acid sequence (SEQ ID NO: 2; upper case letters) of full length **human** IKK.alpha. subunit of an **IKK** complex. Nucleotide positions are indicated to the right and left of the sequence; the "A" of the ATG encoding the initiator methionine is shown as position 1. Underlined amino acid residues indicate the peptide portions of the protein ("peptide 1" and "peptide 2") that were sequenced and used to design oligonucleotide probes. The asterisk indicates the sequence encoding the STOP codon.

Detailed Description Text - DETX:

The present invention provides isolated nucleic acid molecules encoding polypeptide subunits of **human** serine protein **kinase** complex, the IKB **kinase (IKK)**, which is activated in response to proinflammatory signals and phosphorylates proteins (IKB's) that bind to and inhibit the activity of NF-KB transcription factors. For example, the invention provides an isolated nucleic acid molecule (SEQ ID NO: 1) encoding a full length **human** IKK.alpha. subunit having the amino acid sequence shown as SEQ ID NO: 2 (FIG. 1). In addition, the invention provides an isolated nucleic acid molecule (SEQ ID NO: 14; FIG. 2) encoding a full length **human** IKK.beta. subunit having the amino acid sequence shown as SEQ ID NO: 15 (FIG. 3).

Detailed Description Text - DETX:

IKK.alpha. and IKK.beta. have been designated **IKK** subunits because they are components of an approximately 900 kDa complex having IKB **kinase (IKK)** activity and because they share substantial nucleotide and amino acid sequence homology. As disclosed herein, IKK.alpha. and IKK.beta. are related members of a family of **IKK** catalytic subunits (see FIG. 3). The 900 kDa IKB **kinase** complex can be isolated in a single step, for example, by immunoprecipitation using an antibody specific for an **IKK** subunit or by using metal ion chelation chromatography methods (see Example IV). A 300 kDa **IKK** complex also can be isolated as disclosed herein and has **kinase** activity for an IKB substrate (see Example III).

Detailed Description Text - DETX:

The invention also provides nucleotide sequences that can specifically hybridize to a nucleic acid molecule of the invention. Such hybridizing nucleotide sequences are useful, for example, as probes, which can hybridize to a nucleic acid molecule encoding an **IKK** catalytic subunit and allow the identification of the nucleic acid molecule in a sample. A nucleotide sequence of the invention is characterized, in part, in that it is at least nine nucleotides in length, such sequences being particularly useful as primers for the polymerase chain reaction (PCR), and can be at least fourteen nucleotides in length or, if desired, at least seventeen nucleotides in length, such nucleotide sequences being particularly useful as hybridization probes, although such sequences also can be used for PCR. A nucleotide sequence of the invention can comprise at least six nucleotides 5' to nucleotide position 92 as

shown in SEQ ID NO: 1 (FIG. 1), preferably at least nine nucleotides 5' to position 92, or more as desired, where SEQ ID NO: 1 is shown in the conventional manner from the 5'-terminus (FIG. 1; upper left) to the 3'-terminus. Such nucleotide sequences of the invention are particularly useful in methods of diagnosing a pathology, for example, a **human** disease, characterized by aberrant **IKK** activity. For convenience, such nucleotide sequences can comprise a kit, which can be made commercially available and can provide a standardized diagnostic assay.

Detailed Description Text - DETX:

A nucleic acid molecule encoding an IKK.alpha. such as the nucleotide sequence shown in SEQ ID NO: 1 diverges from the sequence encoding the **mouse** homolog (GenBank Accession #U12473) in the region encoding amino acid 30. Thus, a nucleotide sequence comprising nucleotides 88 to 90 as shown in SEQ ID NO: 1, which encodes amino acid 30 of **human** IKK.alpha., can be particularly useful, for example, for identifying the presence of a nucleic acid molecule encoding a **human** IKK.alpha. in a sample. Furthermore, based on a comparison of SEQ ID NO: 1 with SEQ ID NO: 14, the skilled artisan readily can select nucleotide sequences that can hybridize with a nucleic acid molecule encoding a **human** IKK.alpha. or a **human** IKK.beta. or both by designing the sequence to contain conserved or non-conserved nucleotide sequences, as desired. For example, selection of a nucleotide sequence that is highly conserved among SEQ ID NO: 1 and SEQ ID NO: 14 can allow the identification of related members of the **IKK** subunit family of proteins. In comparison, selection of a nucleotide sequence that is present, for example, in SEQ ID NO: 14, but that is not present in SEQ ID NO: 1 or that shares only minimal homology can allow identification of the expression of SEQ ID NO: 14 in a cell, irrespective of whether SEQ ID NO: 1 also is expressed in the cell. It should be recognized, however, that a nucleotide sequence of the invention readily is identifiable in comparison to GenBank Accession #U12473 or #U22512 in that a nucleotide sequence of the invention is not the nucleotide sequence of GenBank Accession #U12473 or #U22512.

Detailed Description Text - DETX:

A "suicide" gene also can be incorporated into a vector so as to allow for selective inducible killing of a cell containing the gene. A gene such as the herpes simplex virus thymidine **kinase** gene (TK) can be used as a suicide gene to provide for inducible destruction of such cells. For example, where it is desired to terminate the expression of an introduced nucleic acid molecule encoding **IKK** or an antisense **IKK** subunit molecule in cells containing the nucleic acid molecule, the cells can be exposed to a drug such as acyclovir or gancyclovir, which can be administered to an individual.

Detailed Description Text - DETX:

The present invention provides an isolated IKB **kinase (IKK)**, including isolated full length **IKK** catalytic subunits. For example, the invention provides an

isolated 300 kDa or 900 kDa complex, which comprises an IKK.alpha. or an IKK.beta. subunit and has IKB **kinase** activity (see Examples I, III and IV). In addition, the invention provides is an isolated **human** IKK.alpha. catalytic subunit (SEQ ID NO: 2; Example II), which contains a previously undescribed N-terminal amino acid sequence and essentially the C-terminal region of **human** CHUK (Connelly and Marcu, supra, 1995) and phosphorylates IKB.alpha. on Ser-32 and Ser-36 and IKB.beta. on Ser-19 and Ser-23 (DiDonato et al., supra, 1996; see, also, Regnier et al., supra, 1997). The invention also provides an isolated IKK.beta. catalytic subunit (SEQ ID NO: 15; Example III), which shares greater than 50% amino acid sequence identity with IKK.alpha., including conserved homology in the **kinase** domain, helix-loop-helix domain and leucine zipper domain.

Detailed Description Text - DETX:

As used herein, the term "isolated," when used in reference to an IKB **kinase** complex or to an **IKK** catalytic subunit of the invention, means that the complex or the subunit is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with an **IKK** in a cell. An isolated 900 kDa IKB **kinase** complex or 300 kDa complex can be isolated, for example, by immunoprecipitation using an antibody that binds to an **IKK** catalytic subunit (see Examples III and IV). In addition, an isolated **IKK** subunit can be obtained, for example, by expression of a recombinant nucleic acid molecule such as SEQ ID NO: 1 or SEQ ID NO: 14, or can be isolated from a cell by a method comprising affinity chromatography using ATP or IKB as ligands (Example I) or using an anti-**IKK** subunit antibody. An isolated **IKK** complex or **IKK** subunit comprises at least 30% of the material in a sample, generally about 50% or 70% or 90% of a sample, and preferably about 95% or 98% of a sample, as described above with respect to nucleic acids.

Detailed Description Text - DETX:

The amino acid sequences for MEKK1 (GenBank Accession # U48596; locus RNU48596), PKR (GenBank Accession # M35663; locus HUMP68A) and CKII (GenBank Accession # M55268 J02924; locus HUMA1CKII) are different from the sequences of the **IKK** subunits disclosed herein (SEQ ID NO: 2 and SEQ ID NO: 15) and, therefore, are distinguishable from the present invention. In addition, a full length **human** IKK.alpha. of the invention is distinguishable from the partial **human** CHUK polypeptide sequence in that the partial **human** CHUK polypeptide (Connelly and Marcu, supra, 1995; GenBank Accession #22512) lacks amino acids 1 to 31 as shown in SEQ ID NO: 2. As disclosed herein, a polypeptide having the amino acid sequence of the partial **human** CHUK polypeptide does not have IKB **kinase** activity when expressed in a cell, indicating that some or all of amino acid residues 1 to 31 are essential for **kinase** activity.

Detailed Description Text - DETX:

A full length **IKK** catalytic subunit of the invention is exemplified by **human** IKK.alpha., which has an apparent molecular mass of about 85 kDa and

phosphorylates IKB.alpha. on Ser-32 and Ser-36. An **IKK** catalytic subunit of the invention also is exemplified by IKK.beta., which is an 87 kDa polypeptide that shares substantial amino acid sequence homology with IKK.alpha. (FIG. 3). As used herein, the term "full length," when used in reference to an **IKK** subunit of the invention, means a polypeptide having an amino acid sequence of an **IKK** subunit expressed normally in a cell. Such a normally expressed **IKK** polypeptide begins with a methionine residue at its N-terminus (Met-1; FIG. 3), the Met-1 being encoded by the initiator ATG (AUG) codon, and ends as a result of the termination of translation due to the presence of a STOP codon. A full length **human IKK** catalytic subunit can be a native **IKK** polypeptide, which is isolated from a cell, or can be produced using recombinant DNA methods such as by expressing the nucleic acid molecule shown as SEQ ID NO: 1 or SEQ ID NO: 14.

Detailed Description Text - DETX:

A peptide portion of an **IKK** subunit can comprise the **kinase** domain of the **IKK** subunit and, therefore, can have the ability to phosphorylate an IKB protein. For example, a peptide portion of SEQ ID NO: 2 comprising amino acids 15 to 301 has the characteristics of a serine-threonine protein **kinase** domain (Hanks and Quinn, Meth. Enzymol. 200:38-62 (1991), which is incorporated herein by reference). Such a peptide portion of an **IKK** subunit can be examined for **kinase** activity by determining that it can phosphorylate IKB.alpha. at Ser-32 and Ser-36 or IKB.beta. at Ser-19 and Ser-23, using methods as disclosed herein. In addition, a peptide portion of an **IKK** subunit can comprise an immunogenic amino acid sequence of the polypeptide and, therefore, can be useful for eliciting production of an antibody that can specifically bind the **IKK** subunit or to an **IKK** complex comprising the subunit, particularly to an epitope comprising amino acid residue 30 as shown in SEQ ID NO: 2 or to an epitope of SEQ ID NO: 15, provided said epitope is not present in a CHUK protein. Accordingly, the invention also provides anti-**IKK** antibodies, which specifically bind to an epitope of an **IKK** complex, particularly an **IKK** catalytic subunit, and to **IKK** subunit binding fragments of such antibodies. In addition, the invention provides cell lines producing anti-**IKK** antibodies or **IKK**-binding fragments of such antibodies.

Detailed Description Text - DETX:

An anti-**IKK** antibody of the invention can be raised using an isolated **IKK** subunit or a peptide portion thereof and can bind to a free, uncomplexed form of **IKK** subunit or can bind to **IKK** subunit when it is associated with a 300 kDa or 900 kDa **IKK** complex. In addition, an anti-**IKK** antibody of the invention can be raised against an isolated 300 kDa or 900 kDa IKB **kinase** complex, which can be obtained as disclosed herein. For convenience, an antibody of the invention is referred to generally herein as an "anti-IKB **kinase** antibody" or an "anti-**IKK** antibody." However, the skilled recognize that the various antibodies of the invention will have unique antigenic specificities, for example, for a free or complexed **IKK** subunit, or both, or for a 300 kDa or 900 kDa IKB **kinase** complex, or both.

Detailed Description Text - DETX:

Anti-**IKK** antibodies can be raised using as an immunogen an isolated full length **IKK** catalytic subunit, which can be prepared from natural sources or produced recombinantly, or a peptide portion of an **IKK** subunit as defined herein, including synthetic peptides as described above. A non-immunogenic peptide portion of an **IKK** catalytic subunit can be made immunogenic by coupling the hapten to a carrier molecule such bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), or by expressing the peptide portion as a fusion protein. Various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane, supra, 1988). It is recognized that, due to the apparently high amino acid sequence identity of the full length **human** IKK.alpha. and **mouse** CHUK, the amino acid sequences of IKK.alpha. polypeptides, as well as IKK.beta. polypeptides, likely are highly conserved among species, particularly among mammalian species. However, antibodies to highly conserved proteins have been raised successfully, for example, in chickens. Such a method can be used to obtain an antibody to an **IKK** subunit, if desired.

Detailed Description Text - DETX:

Particularly useful antibodies of the invention include antibodies that bind with the free, but not the complexed, form of an **IKK** subunit or, alternatively, with the complexed, but not free, form of an **IKK** subunit. Antibodies of the invention also include antibodies that bind with the 300 kDa IKB **kinase** complex or the 900 kDa IKB **kinase** complex or both. It should be recognized, however, that an antibody specific for the 300 kDa or 900 kDa IKB **kinase** complex need not recognize an **IKK** subunit epitope in order to be encompassed within the claimed invention, since, prior to the present disclosure, the 300 kDa and 900 kDa **IKK** complexes were not known (see DiDonato et al., Nature 388:548-554 (1997)).

Detailed Description Text - DETX:

Antibodies of the invention that bind to an activated **IKK** but not to an inactive **IKK,** and, conversely, those that bind to an inactive form of the **kinase** but not to the activated form also are particularly useful. For example, an **IKK** can be activated by phosphorylation of an **IKK** subunit and, therefore, an antibody that recognizes the phosphorylated form of the **IKK,** but that does not bind to the unphosphorylated form can be obtained. In addition, **IKK** can be activated by release of a regulatory subunit and, therefore, an antibody that recognizes a form of the **IKK** complex that is not bound to the regulatory subunit can be obtained. Such antibodies are useful for identifying the presence of active **IKK** in a cell.

Detailed Description Text - DETX:

An anti-**IKK** antibody is useful, for example, for determining the presence or level of an **IKK or of an IKK** subunit in a tissue sample, which can be a lysate

or a histological section. The identification of the presence or level of an **IKK or an IKK** subunit in the sample can be made using well known immunoassay and immunohistochemical methods (Harlow and Lane, supra, 1988). An anti-**IKK** antibody also can be used to substantially purify an IKB **kinase or an IKK** subunit from a sample. In addition, an anti-**IKK** antibody can be used in a screening assay to identify agents that alter the activity of an IKB **kinase**.

Detailed Description Text - DETX:

A kit incorporating an anti-**IKK** antibody, which can be specific for the active or inactive form of IKB **kinase** or can bind to an **IKK** complex or to an **IKK** subunit, regardless of the activity state, can be particularly useful. Such a kit can contain, in addition to an anti-**IKK** antibody, a reaction cocktail that provides the proper conditions for performing the assay, control samples that contain known amounts of an **IKK or IKK** subunit and, if desired, a second antibody specific for the anti-**IKK** antibody. Such an assay also should include a simple method for detecting the presence or amount of an **IKK or an IKK** subunit in a sample that is bound to the anti-**IKK** antibody.

Detailed Description Text - DETX:

Following contact, for example, of a labeled antibody with a sample such as a tissue homogenate or a histological section of a tissue, specifically bound labeled antibody can be identified by detecting the particular moiety. Alternatively, a labeled second antibody can be used to identify specific binding of an unlabeled anti-**IKK** antibody. A second antibody generally will be specific for the particular class of the first antibody. For example, if an anti-IKB **kinase** antibody is of the IgG class, a second antibody will be an anti-IgG antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labeled using a detectable moiety as described above. When a sample is labeled using a second antibody, the sample is first contacted with a first antibody, which is an anti-**IKK** antibody, then the sample is contacted with the labeled second antibody, which specifically binds to the anti-**IKK** antibody and results in a labeled sample.

Detailed Description Text - DETX:

Methods for raising polyclonal antibodies, for example, in a rabbit, goat, **mouse** or other mammal, are well known in the art (see Example V). In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (Harlow and Lane, supra, 1988). Essentially, spleen cells from a **mouse** immunized with an **IKK** complex or an **IKK** subunit or peptide portion thereof can be fused to an appropriate myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using a labeled **IKK** subunit to identify clones that secrete anti-**IKK** monoclonal antibodies. Hybridomas expressing anti-**IKK** monoclonal antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of the antibodies, which are useful, for example, for preparing standardized kits as described above. Similarly, a recombinant phage that

expresses, for example, a single chain anti-**IKK** also provides a monoclonal antibody that can used for preparing standardized kits.

Detailed Description Text - DETX:

A monoclonal anti-**IKK** antibody can be used to prepare anti-idiotypic antibodies, which present an epitope that mimics the epitope recognized by the monoclonal antibody used to prepare the anti-idiotypic antibodies. Where the epitope to which the monoclonal antibody includes, for example, a portion of the **IKK** catalytic subunit **kinase** domain, the anti-idiotypic antibody can act as a competitor of IKB and, therefore, can be useful for reducing the level of phosphorylation of IKB and, consequently, the activity of NF-KB.

Detailed Description Text - DETX:

The present invention further provides methods of identifying an agent that can alter the association of an **IKK** catalytic subunit with a second protein, which can be an upstream activator, a downstream effector such as IKB, an interacting regulatory protein of the **IKK** subunit, or an interacting subunit associated with the 300 kDa or 900 kDa IKB **kinase** complex. As used herein, the term "associate" or "association," when used in reference to an **IKK** subunit and a second protein means that the **IKK** subunit and the second protein have a binding affinity for each other such that they form a bound complex in vivo or in vitro, including in a cell in culture or in a reaction comprising substantially purified reagents. For convenience, the term "bind" or "interact" is used interchangeably with the term "associate."

Detailed Description Text - DETX:

As used herein, the term "second protein" refers to a protein that specifically associates with an **IKK** subunit ("first protein"). Such a second protein is exemplified herein by IKB proteins, including IKB.alpha. and IKB.beta., which are substrates for IKB **kinase** activity and are downstream of the IKB **kinase** in a signal transduction pathway that results in the regulated expression of a gene. In addition, such second proteins are exemplified by the proteins that, together with the **IKK** subunits, form a 300 kDa or 900 kDa IKB **kinase** complex, which coimmunoprecipitates using an anti-**IKK** antibody (see Example IV). Furthermore, since **IKK** subunits such as IKK.alpha. and IKK.beta. interact with each other to form homodimers or heterodimers, a second protein also can be a second **IKK** subunit, which can be the same as or different from the "first" protein.

Detailed Description Text - DETX:

Agents that alter the association of an IKK catalytic subunit and a second protein such as IKB protein or an **IKK** regulatory subunit can be extremely valuable, for example, for limiting excessive cytokine expression as occurs in an acute phase response by preventing the activation of NF-KB, thereby

preventing NF-KB mediated induction of cytokine gene expression. Where, in a drug screening assay of the invention, the second protein is an IKB, the **IKK** subunit can be any protein involved in IKB **kinase** activity, including, for example, **mouse** CHUK (Connelly and Marcu, supra, 1995; GenBank Accession #12473), which, prior to the present disclosure, was not known to have the ability to associate with IKB or to have IKB **kinase** activity.

Detailed Description Text - DETX:

In addition, a second protein can be a protein that is upstream of IKB **kinase** in a signal transduction pathway and associates with the **IKK** complex, particularly with an **IKK** catalytic subunit of the **IKK** complex. Such a second protein, which can be an upstream activator of the IKB **kinase,** can be identified using routine methods for identifying protein-protein interactions as disclosed herein. Such second proteins can be, for example, MEKK1 or PKR or CKII, each of which has been reported to be involved in a pathway leading to phosphorylation of IKB and activation of NF-KB, but neither of which has the characteristics expected of the common IKB **kinase** present at the point where the various NF-KB activation pathways converge (see, for example, Lee et al., supra, 1997), or can be the NF-KB-inducing **kinase** (NIK), which reportedly is upstream from **IKK** in an NF-KB activation pathway (Regnier et al., supra, 1997; Malinin et al., Nature 385:540-544 (1997)).

Detailed Description Text - DETX:

As disclosed herein, two copurifying proteins were isolated by ATP and IKB affinity chromatography and identified by SDS-PAGE (Example I). Partial amino acid sequences were determined and cDNA molecules encoding the proteins were obtained (see Examples I, II and III). One of the proteins has an apparent molecular mass of 85 kDa. Expression in a cell of a cDNA molecule encoding the 85 kDa protein resulted in increased NF-KB activity following cytokine induction as compared to control cells, whereas expression of the antisense of this cDNA decreased the basal NF-KB activity in the cells and prevented cytokine induction of NF-KB activity. Immunoprecipitation of the 85 kDa protein resulted in isolation of the **IKK** complex, the **kinase** activity of which was stimulated rapidly in response to TNF or to IL-1. Based on these functional analyses, the 85 kDa protein was determined to be a component of the 900 kDa IKB **kinase** complex and has been designated IKK.alpha. (SEQ ID NO: 2). The second protein, which copurified with the 85 kDa IKB **kinase,** has an apparent molecular mass of 87 kDa and shares greater than 50% amino acid sequence identity with IKK.alpha. and has been designated IKK.beta. (SEQ ID NO: 15).

Detailed Description Text - DETX:

The ability of the 85 kDa and 87 kDa IKK subunits to associate with other proteins such as a regulatory subunit as well as with IKB is suggested, for example, by the presence in the IKB **kinase** of two different protein binding domains, a helix-loop-helix domain and a leucine zipper domain (see Connelly

and Marcu, supra, 1995; see, also, FIG. 3). While the leucine zipper motif mediates homotypic and heterotypic interactions between IKK.alpha. and IKK.beta., the helix-loop-helix motif serves as a binding site for regulatory proteins necessary for IKB **kinase** activation.

Detailed Description Text - DETX:

A screening assay of the invention also is useful for identifying agents that directly alter the activity of an **IKK**. While such an agent can act, for example, by altering the association of an **IKK** complex or **IKK** catalytic subunit with a second protein, the agent also can act directly as a specific activator or inhibitor of **IKK** activity. Specific protein **kinase** inhibitors include, for example, staurosporin, the heat stable inhibitor of cAMP-dependent protein **kinase,** and the MLCK inhibitor, which are known in the art and commercially available. A library of molecules based, generally, on such inhibitors or on ATP or adenosine can be screened using an assay of the invention to obtain agents that desirably modulate the activity of an **IKK** complex or an **IKK** subunit.

Detailed Description Text - DETX:

As disclosed herein, **IKK** activity can be measured by identifying phosphorylation, for example, of IKB.alpha., either directly or using an antibody specific for the Ser-32 and Ser-36 phosphorylated form of IKB.alpha.. An antibody that binds to IKB.alpha. that is phosphorylated on Ser-32, for example, can be purchased from a commercial source (New England Biolabs; Beverly Mass.). Cultured cells can be exposed to various agents suspected of having the ability to directly alter **IKK** activity, then aliquots of the cells either are collected or are treated with a proinflammatory stimulus such as a cytokine, and collected. The collected cells are lysed and the **kinase** is immunoprecipitated using an anti-**IKK** antibody. A substrate such as IKB.alpha. or IKB.beta. is added to the immunocomplex and the ability of the **IKK** to phosphorylate the substrate is determined as described above. If desired, the anti-**IKK** antibody first can be coated onto a plastic surface such as in 96 well plates, then the cell lysate is added to the wells under conditions that allow binding of **IKK** by the antibody. Following washing of the wells, **IKK** activity is measured as described above. Such a method is extremely rapid and provides the additional advantage that it can be automated for high through-put assays.

Detailed Description Text - DETX:

The specific cellular target upon which a cytokine restraining agent acts has not been reported. However, the myriad of pathologic effects ameliorated by such agents are similar to various pathologies associated with aberrant NF-KB activity, suggesting that cytokine restraining agents may target an effector molecule in a NF-KB signal transduction pathway. Thus, one potential target of a cytokine restraining agent can be an IKB **kinase, particularly an IKK** catalytic subunit of the **kinase**. Accordingly, a screening assay of the invention can be used to determine whether a cytokine restraining agent alters

the activity of IKB **kinase** or alters the association of an **IKK** and a second protein such as IKB. If it is determined that a cytokine restraining agent has such an effect, the screening assay then can be used to screen a library of cytokine regulatory agents to identify those having desirable characteristics, such as those having the highest affinity for the **IKK**.

Detailed Description Text - DETX:

The skilled artisan will recognize that a ligand such as ATP or an IKB or an anti-**IKK** antibody also can be immobilized on various other matrices, including, for example, on magnetic beads, which provide a rapid and simple method of obtaining a fraction containing an ATP- or an IKB-bound **IKK** complex or **IKK** subunit or an anti-IKB **kinase**-bound **IKK** from the remainder of the sample. Methods for immobilizing a ligand such as ATP or an IKB or an antibody are well known in the art (Haystead et al., Eur. J. Biochem. 214:459-467 (1993), which is incorporated herein by reference; see, also, Hermanson, supra, 1996). Similarly, the artisan will recognize that a sample containing an **IKK** complex or an **IKK** subunit can be a cell, tissue or organ sample, which is obtained from an animal, including a mammal such as a **human,** and prepared as a lysate; or can be a bacterial, insect, yeast or mammalian cell lysate, in which an **IKK** catalytic subunit is expressed from a recombinant nucleic acid molecule. As disclosed herein, a recombinantly expressed IKK.alpha. or IKK.beta. such as a tagged IKK.alpha. or IKK.beta. associates into an active 300 kDa and 900 kDa **IKK** complex (see Examples III and IV).

Detailed Description Text - DETX:

Identification and Characterization of a **Human** IKB **Kinase** Complex and **IKK** Subunits

Detailed Description Text - DETX:

Kinase assays were performed using GST fusion proteins containing amino acid residues 1 to 54 of IKB. The fusion proteins were linked to glutathione SEPHAROSE and the beads were used directly in the assays. At earlier stages in the purification of the IKK activity, the beads were washed prior to loading onto the gel to minimize contributions from other proteins. In some of the later characterization of highly purified material, soluble fusion protein was used.

Detailed Description Text - DETX:

Three distinct substrates for the **IKK** activity were used: 1) substrate "WT" contained amino acid residues 1 to 54 of IKB.alpha.; 2) substrate "AA" contained amino acid residues 1 to 54 of IKB.alpha., except that Ser-32 (S32) and S36 were replaced with Ala-32 (A32) and A36, respectively; and 3) substrate "TT" contained amino acid residues 1 to 54 of IKB.alpha., except that S32 and S36 were replaced with Thr-32 (T32) and T36, respectively (DiDonato et al.,

Mol. Cell. Biol. 16:1295-1304 (1996)). Each substrate was expressed as a GST fusion protein. The physiologic, inducible IKB **kinase** is specific for S32 and S36 (WT) in IKB.alpha., but does not recognize the TT or AA mutants (DiDonato et al., Mol. Cell. Biol. 16:1295-1304 (1996)).

Detailed Description Text - DETX:

Supernatant was collected and centrifuged at 38,000 rpm for 80 min in a Beckman 50.1 Ti rotor at 4.degree. C. The supernatant (S100 fraction) was quick frozen in liquid nitrogen and stored at -80.degree. C. Small aliquots of S100 material, prepared from either unstimulated HeLa cells or from TNF.alpha. stimulated cells, were purified in a single passage over a SUPEROSE 6 gel filtration column (1.0.times.30 cm; Pharmacia; Uppsalla Sweden) equilibrated in Buffer A containing 0.1% Brij-35 and 300 mM NaCl and eluted at a flow rate of 0.3 ml/min. 0.6 ml fractions were collected and **kinase** assays were performed on an aliquot of each fraction. The high molecular weight material (fractions 16-20) contained TNF.alpha.-inducible **IKK** activity, which is specific for the WT substrate.

Detailed Description Text - DETX:

110 ml of S100 material (900 mg of protein; Bio-Rad Protein Assay) was pumped onto a Q-SEPHAROSE FAST FLOW column (56 ml bed volume, 2.6 cm ID) equilibrated at 2 ml/min with Buffer A containing 0.1% Brij-35. After the sample was loaded, the column was washed with 100 ml of Buffer A containing 0.1% Brij-35 and 100 mM NaCl, then a linear NaCl gradient was run from 100-300 mM. The gradient volume was 500 ml and the flow rate was 2 ml/min. Ten ml fractions were collected and the **kinase** assay was performed on those fractions that eluted during the gradient. Fractions corresponding to the TNF.alpha.-inducible **IKK** activity (fractions 30-42; i.e., 20-32 of the gradient portion) were pooled. The pooled material contained 40 mg of protein.

Detailed Description Text - DETX:

Since the 85 kDa IKK.alpha. band identified by the **kinase** assay following the above procedure contained only about 10% of the total purified protein, three additional criteria were used to confirm that the identified band was an intrinsic component of the **IKK** complex.

Detailed Description Text - DETX:

In one procedure, the elution profile of the SUPEROSE 6 column was analyzed by silver stained 8% SDS-PAGE gels, then compared to the **kinase** activity profile. For this analysis, 0.3 ml fractions were collected from the SUPEROSE 6 column, then separated by 8% SDS-PAGE and silver stained. This comparison confirmed that a single band of 85 kDa correlated precisely with the elution of **IKK** activity.

Detailed Description Text - DETX:

In a third procedure, purified **IKK** was treated with excess phosphatase, which inactivates the **IKK,** then reactivated by addition of a semi-purified HeLa extract. Phosphatase inactivation was performed by adding excess protein phosphatase 2A catalytic domain (PP2A) to purified IKB **kinase** in 50 mM Tris (pH 7.6), 50 mM NaCl, 1 mM MgCl.sub.2, then equilibrating the reaction for 60 min at 30.degree. C. 1.25 .mu.M okadaic acid was added to completely inactivate the phosphatase and the phosphatase inactivated material was used in standard **kinase** assays and to perform the reactivation and phosphorylation procedure.

Detailed Description Text - DETX:

Cytoplasmic extract was prepared using HeLa S3 cells. The cells were stimulated with TNF.alpha. for 5 min, then harvested in lysis buffer containing 0.1% NP-40 and 0.15 M NaCl. Reactivation was performed at 30.degree. C. in **kinase** buffer for 60 min in the absence of (.gamma.-.sup.32 P)ATP. Samples containing only cold ATP were used for **kinase** activity assays. Reactivation by the HeLa cell extract was performed in the presence of (.gamma.-.sup.32 P)ATP, then the sample was separated by 86 SDS-PAGE and examined by autoradiography. A band of approximately 86 kDa was phosphorylated in the reactivated material and, associated with the reactivation procedure, was restoration of the **IKK** activity.

Detailed Description Text - DETX:

Comparison of the amino acid sequences of IKK.alpha. and IKK.beta. revealed greater than 50% amino acid identity (FIG. 3). In addition, SEQ ID NO: 15 contains a **kinase** domain, which shares 65% amino acid identity with IKK.alpha., a leucine zipper and a helix-loop-helix domain. Based on the sequence homology and domain structure, the polypeptide (SEQ ID NO: 15) was determined to be a member of the **IKK** catalytic subunit family of proteins with IKK.alpha. and, therefore, was designated IKK.beta..

Detailed Description Text - DETX:

The response of IKK.beta.-associated **kinase** activity to various stimuli also was examined in HeLa cells transiently transfected with the HA-IKK.beta. expression vector. After 24 hr, the cells were stimulated with either 10 ng/ml IL-1, 20 ng/ml TNF or 100 ng/ml TPA, then HA-IKK.beta. immune complexes were isolated by immunoprecipitation and **IKK** activity was measured. TNF and IL-1 potently stimulated IKK.beta.-associated **kinase** activity, whereas the response to TPA was weaker. The kinetics of IKK.beta. activation by either TNF or IL-1 essentially were identical to the kinetics of activation of the IKK.alpha.-associated IKB kinase measured by a similar protocol.

Detailed Description Text - DETX:

The levels of **IKK** activities associated with IKK.alpha. and IKK.beta. were compared more precisely by transfecting 293 cells with increasing amounts of HA-IKK.alpha. or HA-IKK.beta. expression vectors (0.1 to 0.5 .mu.g/10.sup.6 cells) and determining the **kinase** activities associated with the two proteins in cell lysates prepared before or after TNF stimulation (20 ng/ml, 5 min); GST-IKB.alpha. (1-54) was used as substrate. The level of expression of each protein was determined by immunoblot analysis and used to calculate the relative levels of specific **IKK** activity.

Detailed Description Text - DETX:

The relative contribution of IKK.alpha. and IKK.beta. to **IKK** activity was examined by constructing mutant subunits in which the lysine (K) codon present at position 44 of each subunit was substituted with a codon for either methionine (M) or alanine (A) codon, respectively. Similar mutations in other protein **kinases** render the enzymes defective in binding ATP and, therefore, catalytically inactive (Taylor et al., Ann. Rev. Cell Biol. 8:429-462 (1992)). The activity of the **IKK** mutants was compared to the activity of their wild type (wt) counterparts by cell-free translation in reticulocyte lysates using GST-IKB.alpha. (1-54) as a substrate. Translation of IKK.alpha. (KM) resulted in formation of IKB **kinase** having only slightly less activity than the **IKK** formed by translation of wt IKK.alpha.. In comparison, translation of IKK.beta. (KA) did not generate **IKK** activity. Translation of wt IKK.beta. generated IKB **kinase** activity as expected.

Detailed Description Text - DETX:

Expression of wt HA-IKK.alpha. generated substantial **IKK** activity that was isolated by immunoprecipitation with anti-HA, whereas very little **IKK** activity was generated in cells transfected with either the HA-IKK.alpha. (LZ).sup.- or HA-IKK.alpha. (HLH).sup.- mutant. Coexpression of the mutant **IKK** subunits with Flag-IKK.beta. resulted in a substantial increase in the **IKK** activity isolated by immunoprecipitation of HA-IKK.alpha., but had no effect on the very low activity that coprecipitated with HA-IKK.alpha. (LZ).sup.-. However, coexpression of Flag-IKK.beta. did stimulate the low level of **IKK** activity associated with HA-IKK.alpha. (HLH).sup.-. Probing of the HA immune complexes with anti-Flag (M2) antibodies indicated that both wt HA-IKK.alpha. and HA-IKK.alpha. (HLH).sup.- associated with similar amounts of Flag-IKK.beta., but that the HA-IKK.alpha. (LZ).sup.- mutant did not associate with Flag-IKK.beta.. These results indicate that the lower IKB **kinase** activity associated with the IKK.alpha. (LZ).sup.- mutant is due to a defect in its ability to interact with IKK.beta.. The lower IKB **kinase** activity of the IKK.alpha. (HLH).sup.- mutant, on the other hand, likely is due to a defect in the ability to interact with a second, undefined protein, since the HLH mutant can interact with IKK.beta..

Detailed Description Text - DETX:

pRC-HF-IKK.alpha. was transfected into **human** embryonic kidney 293 cells and transfected cells were selected for growth in the presence of G418. A low basal level of **IKK** activity was detected in cells expressing HF-IKK.alpha. and **IKK** activity increased several fold when the cells were treated with TNF.alpha.. This result indicates that the HF-IKK.alpha. expression in 293 cells is associated with **IKK** activity in the cells and that such **IKK** activity is inducible in response to TNF.alpha..

Detailed Description Text - DETX:

The second protein, which can be IKB or a protein that copurifies with **IKK** subunit as part of the 900 kDa IKB **kinase,** for example, can be detectably labeled with a moiety such as a fluorescent molecule or a radiolabel (Hermanson, supra, 1996), then contacted in solution with the immobilized **IKK** subunit under conditions as described in Example I, which allow IKB to specifically associate with the **IKK** subunit. Preferably, the reactions are performed in 96 well plates, which allow automated reading of the reactions. Various agents such as drugs then are screened for the ability to alter the association of the **IKK** subunit and IKB.

US-PAT-NO: 6235513

DOCUMENT-IDENTIFIER: US 6235513 B1

TITLE: IKK-.alpha. proteins, nucleic acids and methods

DATE-ISSUED: May 22, 2001

INVENTOR-INFORMATION:

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APPL-NO: 09/ 023324

DATE FILED: February 13, 1998

PARENT-CASE:

This a divisional application of U.S. Ser. No. 08/890,854, filed Jul. 10, 1997, which is a continuing application under 35USC120 of U.S. Ser. No. 08/887,115 filed Jul. 1, 1997, abandoned.

US-CL-CURRENT: 435/194

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

36 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **human kinase** I.kappa.B **Kinase, IKK**-.alpha., as a NIK-interacting protein. **IKK**-.alpha. has sequence similarity to the conceptual translate of a previously identified open reading frame (SEQ ID NO:5) postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.alpha. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.alpha. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.alpha. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.alpha. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.alpha.-specific structure and activity and modulators of **IKK**-.alpha. function, particularly I.kappa.B **kinase** activity. **IKK**-.alpha. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.alpha. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. gene, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.alpha. transcripts), therapy (e.g. **IKK**-.alpha. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a human **IKK**-.alpha. polypeptide is shown as SEQ ID NO:3, and the full conceptual translate is shown as SEQ ID NO:4. The **IKK**-.alpha. polypeptides of the invention include incomplete translates of SEQ ID NO:3 which translates and deletion mutants of SEQ ID NO:4 have **human IKK**-.alpha.-specific amino acid sequence, binding

specificity or function and comprise at least one of Cys30, Leu604, Thr679, Ser680, Pro684, Thr686 and Ser678. Preferred translates/deletion mutants comprise at least a 6 residue Cys30, Leu604, Thr679, Ser680, Pro684, Thr686 or Ser687-containing domain of SEQ ID NO:4, preferably including at least 8, more preferably at least 12, most preferably at least 20 contiguous residues which immediately flank said residue, with said residue preferably residing within said contigous residues, see, e.g. Table I; which mutants provide hIKK-.alpha. specific epitopes and immunogens.

Brief Summary Text - BSTX:

The subject domains provide **IKK**-.alpha. domain specific activity or function, such as **IKK**-.alpha.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)). As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

IKK-.alpha.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.alpha. polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.alpha. substrate, a IKK-.alpha. regulating protein or other regulator that directly modulates IKK-.alpha. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.alpha. specific agent such as those identified in screening assays such as described below. IKK-.alpha.-binding specificity may assayed by kinase activity or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.alpha.-expressing cells, to elicit IKK-.alpha. specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the IKK-.alpha. binding specificity of the subject IKK-.alpha. polypeptides necessarily distinguishes the murine and human CHUK sequences of Connelly and Marcu (1995) as well as IKK-.beta. (SEQ ID NO:4).

Brief Summary Text - BSTX:

The claimed **IKK**-.alpha. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is

associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The **IKK**-.alpha. polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, N.Y.) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to **IKK** polypeptides, preferably the claimed **IKK**-.alpha. polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-specific binding agents include **IKK**-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK** function, e.g. **IKK**-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK** I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK** I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-derived peptide inhibitors, etc., see Tables II and III. **IKK** specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) July 1995;48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry D W et al. Science Aug. 19, 1994;19;265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science Mar. 24, 1995;267(5205):1782-8). Additional

IKK inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz AF et al., Proc Natl Acad Sci USA Feb. 28, 1995;92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell Dec. 15, 1995;83(6): 1001-9). Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby O, et al. Proc Natl Acad Sci USA May 24, 1994;91(11):4761-5; Barja P, et al., Cell Immunol January 1994;153(1):28-38; Villar-Palasi C, Biochim Biophys Acta Dec. 30, 1994;1224(3):384-8; Liu WZ, et al., Biochemistry Aug. 23, 1994;33(33):10120-6)

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK** binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK** modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK** interaction with a natural **IKK** binding target, in particular, **IKK** phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development,

Brief Summary Text - BSTX:

vitro binding assays employ a mixture of components including an **IKK** polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK** binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at

least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta. or .epsilon.-derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject **IKK** polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK** polypeptide and one or more binding targets is detected by any convenient way. For **IKK kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.alpha. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Text - BSTX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic .alpha.-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Brief Summary Text - BSTX:

Interaction of **IKK**-.alpha. and NIK in **Human** Cells

Brief Summary Text - BSTX:

The interaction of **IKK**-.alpha. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.alpha. containing an N-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK or HA epitope-tagged TRAF proteins. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK or TRAF proteins were detected by immunoblot analysis with an anti-Myc or anti-HA monoclonal antibodies. In this assay, **IKK**-.alpha. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.alpha. protein lacking most of the N-terminal **kinase** domain (**IKK**-.alpha..sub.(307-745)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.alpha. mediates the interaction with NIK. In contrast to NIK, **IKK**-.alpha. failed to associate with either TRAF2 or TRAF3. However, when NIK was coexpressed with **IKK**-.alpha. and TRAF2, strong coprecipitation of TRAF2 with **IKK**-.alpha. was detected, indicating the formation of a ternary complex between **IKK**-.alpha., NIK and TRAF2.

Brief Summary Text - BSTX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.alpha..sub.(307-745) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.alpha..sub.(307-745) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.alpha..sub.(307-745) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.alpha. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.alpha. functions as a common mediator of NF-.kappa.B activation, by TNF and IL-1 downstream of NIK.

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors Inhibitors Citations HA-100.sup.1 1. Hagiwara, M,. et al. Mol. Pharmacol. 32: 7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172: 993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151: 542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135: 397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55: 1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176: 288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et

al. Biochem Biophys Res Com 159: 548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24:497(1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8: 732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39: 1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238: 1726(1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167: 657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525: 198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255: 1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265: 5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142: 436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261: 989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29: 577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23: 5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37: 482 (1990)

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors Inhibitors Citations HA-100.sup.1 1. Hagiwara, M,. et al. Mol. Pharmacol. 32: 7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172: 993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151: 542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135: 397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55: 1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176: 288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159: 548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24:497(1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8: 732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39: 1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238: 1726(1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167: 657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525: 198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255: 1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265: 5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142: 436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261: 989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29: 577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23: 5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37: 482 (1990)

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.alpha. (SEQ ID NO:4) at 20 .mu.g/ml in PBS.

US-PAT-NO: 6235512

DOCUMENT-IDENTIFIER: US 6235512 B1

TITLE: IKK-.alpha. proteins, nucleic acids and methods

DATE-ISSUED: May 22, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Cao; Zhaodan	Pacifica	CA	N/A	N/A
Regnier; Catherine	South San Francisco	CA	N/A	N/A

APPL-NO: 08/ 890854

DATE FILED: July 10, 1997

PARENT-CASE:

This a continuing application under 35USC120 of U.S. Ser. No. 08/887,115 filed Jul. 1, 1997, abandoned.

US-CL-CURRENT: 435/194; 435/325 ; 536/23.2

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

17 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B

kinase, IKK-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **human kinase** I.kappa.B **Kinase, IKK**-.alpha., as a NIK-interacting protein. **IKK**-.alpha. has sequence similarity to the conceptual translate of a previously identified open reading frame (SEQ ID NO:5) postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.alpha. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK over expression; transiently expressed **IKK**-.alpha. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.alpha. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.alpha. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.alpha.-specific structure and activity and modulators of **IKK**-.alpha. function, particularly I.kappa.B **kinase** activity. **IKK**-.alpha. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.alpha. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. gene, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.alpha. transcripts), therapy (e.g. **IKK**-.alpha. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.alpha. polypeptide is shown as SEQ ID NO:3, and the full conceptual translate is shown as SEQ ID NO:4. The **IKK**-.alpha. polypeptides of the invention include incomplete translates of SEQ ID NO:3 which translates and deletion mutants of SEQ ID NO:4 have **human IKK**-.alpha.-specific amino acid sequence, binding specificity or function and comprise at least one of Cys30, Leu604, Thr679,

Ser680, Pro684, Thr686 and Ser678. Preferred translates/deletion mutants comprise at least a 6 residue Cys30, Leu604, Thr679, Ser680, Pro684, Thr686 or Ser687-containing domain of SEQ ID NO:4, preferably including at least 8, more preferably at least 12, most preferably at least 20 contiguous residues which immediately flank said residue, with said residue preferably residing within said contiguous residues, see, e.g. Table I; which mutants provide hIKK-.alpha. specific epitopes and immunogens.

Brief Summary Text - BSTX:

The subject domains provide **IKK**-.alpha. domain specific activity or function, such as **IKK**-.alpha.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)). As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

IKK-.alpha.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.alpha. polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.alpha. substrate, a IKK-.alpha. regulating protein or other regulator that directly modulates IKK-.alpha. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.alpha. specific agent such as those identified in screening assays such as described below. IKK-.alpha.-binding specificity may assayed by kinase activity or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.alpha.-expressing cells, to elicit IKK-.alpha. specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the IKK-.alpha. binding specificity of the subject IKK-.alpha. polypeptides necessarily distinguishes the murine and human CHUK sequences of Connelly and Marcu (1995) as well as IKK-.beta. (SEQ ID NO:4).

Brief Summary Text - BSTX:

The claimed **IKK**-.alpha. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%,

and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The **IKK**-.alpha. polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to **IKK** polypeptides, preferably the claimed **IKK**-.alpha. polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-specific binding agents include **IKK**-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK** function, e.g. **IKK**-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK** I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK** I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-derived peptide inhibitors, etc., see Tables II and III. **IKK** specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiotic (Tokyo) July 1995; 48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry DW et al. Science Aug. 19, 1994; 265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science Mar. 24, 1995; 267(5205):1782-8). Additional **IKK** inhibitors include peptide-based substrate competitors endogenously made by the

mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz AF et al., Proc Natl Acad Sci USA Feb. 28, 1995; 92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell Dec. 15, 1995; 83(6):1001-9). Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby O, et al. Proc Natl Acad Sci USA May 24, 1994; 91(11):4761-5; Baija P, et al., Cell Immunol January 1994; 153(1):28-38; Villar-Palasi C, Biochim Biophys Acta Dec. 30, 1994;1224(3):384-8; Liu WZ, et al., Biochemistry Aug. 23, 1994; 33(33):10120-6).

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK** binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK** modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK** interaction with a natural **IKK** binding target, in particular, **IKK** phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Brief Summary Text - BSTX:

In vitro binding assays employ a mixture of components including an **IKK** polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK** binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately

flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta. or .epsilon. -derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject **IKK** polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK** polypeptide and one or more binding targets is detected by any convenient way. For **IKK kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.alpha. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors Inhibitors Citations HA-100.sup.1 1. Hagiwara, M,. et al. Mol. Pharmacol. 32: 7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172: 993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151: 542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135: 397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55: 1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176: 288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159: 548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24: 497(1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8: 732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39: 1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238: 1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167: 657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525: 198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255: 1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265: 5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142: 436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. 3. Biol. Chem. 261: 989 (1986) KT5926.sup.22 18. Inagaki, M., .et al. Mol. Pharmacol. 29: 577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231: 141 (1984) 20. Hidaka,

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Detailed Description Text - DETX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic .alpha.-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Detailed Description Text - DETX:

Interaction of **IKK**-.alpha. and NIK in **Human** Cells

Detailed Description Text - DETX:

The interaction of **IKK**-.alpha. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.alpha. containing an N-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK or HA epitope-tagged TRAF proteins. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK or TRAF proteins were detected by immunoblot analysis with an anti-Myc or anti-HA monoclonal antibodies. In this assay, **IKK**-.alpha. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.alpha. protein lacking most of the N-terminal **kinase** domain (**IKK**-.alpha..sub.(307-745)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.alpha. mediates the interaction with NIK. In contrast to NIK, **IKK**-.alpha. failed to associate with either TRAF2 or TRAF3. However, when NIK was coexpressed with **IKK**-.alpha. and TRAF2, strong coprecipitation of TRAF2 with **IKK**-.alpha. was detected, indicating the formation of a ternary complex between **IKK**-.alpha., NIK and TRAF2.

Detailed Description Text - DETX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.alpha..sub.(307-745) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.alpha..sub.(307-745) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.alpha..sub.(307-745) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.alpha. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.alpha. functions as a common mediator of NF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.alpha. (SEQ ID NO:4) at 20 .mu.g/ml in PBS.

US-PAT-NO: 6235492

DOCUMENT-IDENTIFIER: US 6235492 B1

TITLE: IKK-.alpha. proteins, nucleic acids and methods

DATE-ISSUED: May 22, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Cao; Zhaodan	Pacifica	CA	N/A	N/A
Regnier; Catherine	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 032476

DATE FILED: February 26, 1998

PARENT-CASE:

This is a divisional application of U.S. Ser. No. 08/890,854, filed Jul. 10, 1997, which is a continuing application under 35USC120 of U.S. Ser. No. 08/887,115 filed Jul. 1, 1997, abandoned.

US-CL-CURRENT: 435/15

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

49 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **human kinase** I.kappa.B **Kinase, IKK**-.alpha., as a NIK-interacting protein. **IKK**-.alpha. has sequence similarity to the conceptual translate of a previously identified open reading frame (SEQ ID NO:5) postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.alpha. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.alpha. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.alpha. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.alpha. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.alpha.-specific structure and activity and modulators of **IKK**-.alpha. function, particularly I.kappa.B **kinase** activity. **IKK**-.alpha. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.alpha. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. gene, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.alpha. transcripts), therapy (e.g. **IKK**-.alpha. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.alpha. polypeptide is shown as SEQ ID NO:3, and the full conceptual translate is shown as SEQ ID NO:4. The **IKK**-.alpha. polypeptides of the invention include incomplete translates of SEQ ID NO:3 which translates and deletion mutants of SEQ ID NO:4 have **human IKK**-.alpha.-specific amino acid sequence, binding

specificity or function and comprise at least one of Cys30, Leu604, Thr679, Ser680, Pro684, Thr686 and Ser678. Preferred translates/deletion mutants comprise at least a 6 residue Cys30, Leu604, Thr679, Ser680, Pro684, Thr686 or Ser687-containing domain of SEQ ID NO:4, preferably including at least 8, more preferably at least 12, most preferably at least 20 contiguous residues which immediately flank said residue, with said residue preferably residing within said contigous residues, see, e.g. Table I; which mutants provide hIKK-.alpha. specific epitopes and immunogens.

Brief Summary Text - BSTX:

The subject domains provide **IKK**-.alpha. domain specific activity or function, such as **IKK**-.alpha.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)). As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.epsilon., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

IKK-.alpha.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.alpha. polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.alpha. substrate, a IKK-.alpha. regulating protein or other regulator that directly modulates IKK-.alpha. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.alpha. specific agent such as those identified in screening assays such as described below. IKK-.alpha.-binding specificity may assayed by kinase activity or binding equilibrium constants(usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.alpha.-expressing cells, to elicit IKK-.alpha. specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the IKK-.alpha. binding specificity of the subject IKK-.alpha. polypeptides necessarily distinguishes the murine and human CHUK sequences of Connelly and Marcu (1995) as well as IKK-.beta. (SEQ ID NO:4).

Brief Summary Text - BSTX:

The claimed **IKK**-.alpha. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is

associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The **IKK**-.alpha. polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to **IKK** polypeptides, preferably the claimed **IKK**-.alpha. polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-specific binding agents include **IKK**-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK** function, e.g. **IKK**-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK** I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK** I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-derived peptide inhibitors, etc., see Tables II and III. **IKK** specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) 1995 July;48(7): 535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry D W et al. Science Aug. 19, 1994;265(5175): 1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science Mar. 24, 1995;267(5205): 1782-8). Additional **IKK**

inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz AF et al., Proc Natl Acad Sci USA Feb. 28, 1995;92(5): 1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell Dec. 15, 1995;83(6): 1001-9). Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby O, et al. Proc Natl Acad Sci USA May 24, 1994;91(11): 4761-5; Barja P, et al., Cell Immunol 1994 January; 153(1):28-38; Villar-Palasi C, Biochim Biophys Acta Dec. 30, 1994;1224(3): 384-8; Liu WZ, et al., Biochemistry Aug. 23, 1994;33(33): 10120-6).

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK** binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK** modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK** interaction with a natural **IKK** binding target, in particular, **IKK** phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Brief Summary Text - BSTX:

In vitro binding assays employ a mixture of components including an **IKK** polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK** binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at

least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta., or .epsilon.-derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject **IKK** polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK** polypeptide and one or more binding targets is detected by any convenient way. For **IKK kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.alpha. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Text - BSTX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic .alpha.-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Brief Summary Text - BSTX:

Interaction of **IKK**-.alpha. and NIK in **Human** Cells

Brief Summary Text - BSTX:

The interaction of **IKK**-.alpha. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.alpha. containing an N-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK or HA epitope-tagged TRAF proteins. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK or TRAF proteins were detected by immunoblot analysis with an anti-Myc or anti-HA monoclonal antibodies. In this assay, **IKK**-.alpha. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.alpha. protein lacking most of the N-terminal **kinase** domain (**IKK**-.alpha..sub.(307-745)) was able to associate with NIK, indicating that the a-helical C-terminal half of **IKK**-.alpha. mediates the interaction with NIK. In contrast to NIK, **IKK**-.alpha. failed to associate with either TRAF2 or TRAF3. However, when NIK was coexpressed with **IKK**-.alpha. and TRAF2, strong coprecipitation of TRAF2 with **IKK**-.alpha. was detected, indicating the formation of a ternary complex between **IKK**-.alpha., NIK and TRAF2.

Brief Summary Text - BSTX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.alpha..sub.(307-745) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.alpha..sub.(307-745) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.alpha..sub.(307-745) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.alpha. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.alpha. functions as a common mediator of NF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors Inhibitors Citations HA-100.sup.1 1. Hagiwara, M., et al. Mol. Pharmacol. 32:7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172:993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151:542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135:397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55:1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176:288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al.

Biochem Biophys Res Com 159:548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24:497 (1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8:732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39:1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238:1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167:657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525:198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255:1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265:5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142:436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261:989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29:577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23:5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37:482 (1990)

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors Inhibitors Citations HA-100.sup.1 1. Hagiwara, M., et al. Mol. Pharmacol. 32:7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172:993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151:542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135:397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55:1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176:288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159:548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24:497 (1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8:732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39:1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238:1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167:657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525:198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255:1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265:5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142:436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261:989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29:577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23:5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37:482 (1990)

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.alpha. (SEQ ID NO:4) at 20 .mu.g/ml in PBS.

US-PAT-NO: 6207458

DOCUMENT-IDENTIFIER: US 6207458 B1

TITLE: Proteins capable of regulating NF-.kappa.B JNK and apoptosis pathways and methods of using the same

DATE-ISSUED: March 27, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chaudhary; Preet M.	Dallas	TX	N/A	N/A
Hood; Leroy	Seattle	WA	N/A	N/A

APPL-NO: 09/ 074044

DATE FILED: May 7, 1998

US-CL-CURRENT: 435/6; 435/18 ; 435/23 ; 435/4 ; 435/40.5 ; 435/40.51 ; 435/40.52 ; 435/7.1 ; 435/7.72

ABSTRACT:

Testing methods are provided for determining whether given candidate compounds are effective for regulating NF-.kappa.B, JNK and apoptosis cell activities. The methods involve forming a mixture including a compound such as a proteinaceous specie containing two death effector domains (DEDs) or structural or functional homologs and analogs thereof, the candidate compound and a binding target capable of specifically binding to at least one of the DEDs. This mixture is incubated under conditions such that, but for the presence of the candidate compound, the cell activity takes place to a determinable extent. After incubation, the activity is determined and is compared with the determinable extent thereof in the absence of the candidate compound. The assays may be carried out intracellularly or in a cell-free assay. Methods for altering NF-.kappa.B, JNK and apoptosis activities in the cell are also provided, and comprise introducing into the cell an activity-regulating amount of a dual DEDs-containing proteinaceous specie.

15 Claims, 44 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 26

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Detailed Description Text - DETX:

All cDNA clones refer to the **human** sequences unless specified otherwise. The sequences and/or expression constructs for cDNAs clones encoding the following molecules have been previously described in the listed references: Caspase 8 (FLICE; GenBank U58143) (Muzio et al., Cell, 85:817-827, 1996); Caspase 7 (ICE-LAP3; GenBank U39613) (Duan et al., J Biol Chem, 271:1621-1625, 1996); Caspase 10 (Mch4; GenBank Q92851) and its active site mutant (i.e. Caspase 10 C358A) (Fernandes-Alnemri et al., Proc Nat'l Acad Sci USA, 93:7464-7469,1996); Caspase 10 (FLICE2) (Vincenz et al., J Biol Chem, 272:6578-6583,1997); Caspase 9 (ICE-LAP6; GenBank P55211) (Duan et al., J Biol Chem, 271:16720-16724, 1996); Caspase 3 (YAMA; GenBank P42574) (Tewari et al., Cell, 81:801-809, 1995); Caspase 2-GenBank P42575 (Wang et al., Cell, 78:739-750,1994); Caspase 1-GenBank P89116 (Thornberry etal., Nature, 356:768-764,1992); FADD-GenBank Q13158 (Chinnaiyan et al., Cell, 81:505-512;1995); E8 (PIR Database S55668) and MC159L (GenBank U60315) (Hu et al., J Biol Chem, 272:9621-96214, 1997); TNFR1 (GenBank P 19438) and DR3 (GenBank U83597) (Chinnaiyan et al., Science, 274:990-992, 1996); crmA, cowpox serpin, (Tewari et al., J Biol Chem, 270:3255-3260, 1995); CD40 (GenBank P25942) (Stamenkovic et al., Embo J, 8:1403-1410,1989); NIK (NF-.kappa.B inducible **kinase,** GenBank Y10256), NIK.DELTA.1234 and NIK.DELTA.2101 (dominant negative NIK) (Natoli et al., J Biol Chem, 272:26079-26082, 1997); mTRAF1 (GenBank P39428) and mTRAF2 (GenBank P39429) (Rothe et al., Cell, 78:681-692,1994); TRAF3 (GenBank U15637) (Cheng et al., Science, 267:1494-1498;1995); mTRAF5 (GenBank D83528) (Nakano et al., J Biol Chem, 271:14661-14664,1996); A20 (Sarma et al., J Biol Chem, 270:12343-12346, 1995); I-TRAF (Rothe et al., Proc Nat'l Acad Sci USA, 93:8241-8246, 1996); **IKK1** (GenBank AF009225, **IKK2** (GenBank AF029684) and their mutants (Nakano et al., Proc Nat'l Acad Sci USA, 95:3537-3542, 1998); DN-I.kappa.B.alpha. or I.kappa.B.alpha.-.DELTA.N (missing the N-terminal 36 amino acids) (Brockman et al., Mol Cell Biol, 15:2809-18;1995); NF-.kappa.B driven luciferase reporter construct (Berberich et al., J Immunol, 153:4357-66;1994); and, JIP (Dickens et al., Science, 277:693-696, 1997). These constructs were either used directly or further modified to add appropriate epitope-tags using PCR and standard molecular biology cloning techniques as described in Molecular Cloning, 2d Ed. by Sambrook, Fritsch, Maniatis, Cold Spring Harbor Laboratory Press, 1989, incorporated by reference herein. An RSV promoter driven .beta.-galactosidase reporter construct was a gift of Dr. Mark Kay (University of Washington).

Detailed Description Text - DETX:

This experiment was conducted to determine whether Caspase 8 can interact with **IKK1** (or IKK.alpha.) and **IKK2** (or IKK.beta.) proteins. **IKK1 and IKK2,** like RIP and NIK, are serine-threonine protein **kinases** involved in the activation of NF-.kappa.B pathway. The co-immunoprecipitation assay followed was similar to that described in Example 24 except the first expression vector consisted of myc-tagged Caspase 8 C360S, and the second expression vector consisted of FLAG-tagged **IKK1 or IKK2** respectively. FLAG-tagged proteins were immunoprecipitated using FLAG beads or control beads. Co-immunoprecipitating myc-Caspase 8 was detected by western blot analysis using a rabbit polyclonal antibody against the myc tag.

Detailed Description Text - DETX:

The results of this experiment are shown in FIG. 40 and demonstrate that Caspase 8 directly interacts with **IKK1 and IKK2** proteins when co-expressed with **IKK1 or IKK2** in mammalian cells. Furthermore, combined with Examples 29, 31, and 33, this example confirms that Caspases and DEDs-containing proteins can interact with serine-threonine protein **kinases**. An assay based on interaction between Caspases and DEDs-containing proteins with **IKK1 or IKK2** can be used as a screening tool for identifying lead compounds for pharmacological agents capable of modulating interactions between Caspases (and/or DEDs-containing proteins) and **IKK1 or IKK2** for the purpose of diagnosis and treatment of diseases associated with the dysfunction of DEDs-containing proteins-IKK1/IKK2 and Caspases-IKK1/IKK2 signal transduction pathways. Similar experiments confirmed that **IKK1** also interacts with MRIT-.alpha.1.

US-PAT-NO: 6172195

DOCUMENT-IDENTIFIER: US 6172195 B1

TITLE: IKAP proteins and methods

DATE-ISSUED: January 9, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Cohen; Lucie	South San Francisco	CA	N/A	N/A
Baeuerle; Patrick	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 286891

DATE FILED: April 6, 1999

PARENT-CASE:

This is a divisional application of U.S. Ser. No. 08/971,244, filed Nov. 16, 1997, now U.S. Pat. No. 5,891,719, which is incorporated herein by reference.

US-CL-CURRENT: 530/350; 436/501 ; 530/300 ; 530/351

ABSTRACT:

The invention provides methods and compositions relating to IKAP proteins which regulate cellular signal transduction and transcriptional activation, and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed IKAP encoding nucleic acids or purified from human cells. The invention provides isolated IKAP hybridization probes and primers capable of specifically hybridizing with the disclosed IKAP genes, IKAP-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

26 Claims, 1 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 1

---------- KWIC ---------

Brief Summary Text - BSTX:

The NF-.kappa.B-inducing **kinase** (NIK) is a member of the MAP **kinase kinase kinase** (MAP3K) family that was identified as a TRAF2-interacting protein (Malinin et al., 1997). NIK activates NF-.kappa.B when overexpressed, and **kinase**-inactive mutants of NIK comprising its TRAF2-interacting C-terminal domain (NIK.sub.(624-947)) or lacking two crucial lysine residues in its **kinase** domain (NIK.sub.(KK429-430AA)) behave as dominant-negative inhibitors that suppress TNF-, IL-1-, and TRAF2-induced NF-.kappa.B activation (Malinin et al., 1997). Recently, NIK was found to associate with additional members of the TRAF family, including TRAF5 and TRAF6. Catalytically inactive mutants of NIK also inhibited TRAF5- and TRAF6-induced NF-.kappa.B activation, thus providing a unifying concept for NIK as a common mediator in the NF-.kappa.B signaling cascades triggered by TNF and IL-1 downstream of TRAFs. Recently two NIK-interacting protein designated characterized as novel **human kinase** I.kappa.B **Kinases, IKK**-.alpha. and IKK.beta. have been reported (Woronicz et al., 1997; Mercurio et al. 1997; Maniatis, 1997). Catalytically inactive mutants of **IKK** suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK** associates with endogenous I.kappa.B.alpha. complex; and **IKK** phosphorylates I.kappa.B.alpha. on serines 32 and 36.

US-PAT-NO: 6160095

DOCUMENT-IDENTIFIER: US 6160095 A

TITLE: Proteins capable of regulating NF-.kappa.B, JNK and apoptosis pathways and methods of using the same

DATE-ISSUED: December 12, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chaudhary; Preet M.	Dallas	TX	N/A	N/A
Hood; Leroy	Seattle	WA	N/A	N/A

APPL-NO: 09/ 382155

DATE FILED: August 24, 1999

PARENT-CASE:

RELATED APPLICATION This is a division of application Ser. No. 09/074,044 filed May 7, 1998.

US-CL-CURRENT: 530/350

ABSTRACT:

Testing methods are provided for determining whether given candidate compounds are effective for regulating NF-.kappa.B, JNK and apoptosis cell activities. The methods involve forming a mixture including a compound such as a proteinaceous specie containing two death effector domains (DEDs) or structural or functional homologs and analogs thereof, the candidate compound and a binding target capable of specifically binding to at least one of the DEDs. This mixture is incubated under conditions such that, but for the presence of the candidate compound, the cell activity takes place to a determinable extent. After incubation, the activity is determined and is compared with the determinable extent thereof in the absence of the candidate compound. The assays may be carried out intracellularly or in a cell-free assay. Methods for altering NF-.kappa.B, JNK and apoptosis activities in the cell are also provided, and comprise introducing into the cell an activity-regulating amount of a dual DEDs-containing proteinaceous specie.

1 Claims, 44 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 26

---------- KWIC ---------

Detailed Description Text - DETX:

All cDNA clones refer to the **human** sequences unless specified otherwise. The sequences and/or expression constructs for cDNAs clones encoding the following molecules have been previously described in the listed references: Caspase 8 (FLICE; GenBank U58143) (Muzio et al., Cell, 85:817-827, 1996); Caspase 7 (ICE-LAP3; GenBank U39613) (Duan et al., J. Biol Chem, 271:1621-1625, 1996); Caspase 10 (Mch4; GenBank Q92851) and its active site mutant (i.e. Caspase 10 C358A) (Fernanes-Alnemri et al., Proc. Nat'l Acad Sci U.S.A., 93:7464-7469, 1996); Caspase 10 (FLICE2) (Vincenz et al., J Biol Chem, 272:6578-6583,1997); . Caspase 9 (ICE-LAP6; GenBank P55211) (Duan et al., J Biol Chem, 271:16720-16724, 1996); Caspase 3 (YAMA; GenBank P42574) (Tewari et al., Cell,. 81:801-809, 1995); Caspase 2--GenBank P42575 (Wang et al., Cell, 78:739-750,1994); Caspase 1--GenBank P89116 (Thornberry et al., Nature,356:768-764,1992); FADD--GenBank Q13158 (Chinnaiyan et al., Cell, 81:505-512; 1995); E8 (PIR Database S55668) and MC159L (GenBank U60315) (Hu et al., J Biol Chem, 272:9621-96214, 1997); TNFR1 (GenBank P19438) and DR3 (GenBank U83597) (Chinnaiyan et al., Science, 274:990-992, 1996); crmA, cowpox serpin, (Tewari et al., J Biol Chem, 270:3255-3260, 1995); CD40 (GenBank P25942) (Stamenkovic et al., Embo J, 8:1403-1410,1989); NIK (NF-.kappa.B-inducible **kinase,** GenBank Y10256), NIK.DELTA.1234 and NIK.DELTA.2101 (dominant negative NIK) (Natoli et al., J Biol Chem, 272:26079-26082, 1997); mTRAF1 (GenBank P39428) and mTRAF2 (GenBank P39429) (Rothe et al., Cell, 78:681-692,1994); TRAF3 (GenBank U15637) (Cheng et al., Science, 267:1494-1498; 1995); mTRAF5 (GenBank D83528) (Nakano et al., J Biol Chem, 271:14661-14664,1996); A20 (Sarma et al., J Biol Chem, 270:12343-12346, 1995); I-TRAF (Rothe et al., Proc Nat'l Acad Sci U.S.A., 93:8241-8246, 1996); **IKK1** (GenBank AF009225, **IKK2** (GenBank AF029684) and their mutants (Nakano et al., Proc Nat'l Acad Sci U.S.A., 95:3537-3542, 1998); DN-I.kappa.B.alpha. or I.kappa.B.alpha.-.DELTA.N (missing the N-terminal 36 amino acids) (Brockman et al., Mol Cell Biol, 15:2809-18; 1995); NF-.kappa.B driven luciferase reporter construct (Berberich et al., J Immunol, 153:4357-66; 1994); and, JIP (Dickens et al., Science, 277:693-696,1997). These constructs were either used directly or further modified to add appropriate epitope-tags using PCR and standard molecular biology cloning techniques as described in Molecular Cloning, 2d Ed. by Sambrook, Fritsch, Maniatis, Cold Spring Harbor Laboratory Press, 1989, incorporated by reference herein. An RSV promoter driven .beta.-galactosidase reporter construct was a gift of Dr. Mark Kay (University of Washington).

Detailed Description Text - DETX:

This experiment was conducted to determine whether Caspase 8 can interact with **IKK1** (or IKK.alpha.) and **IKK2** (or IKK.beta.) proteins. **IKK1 and IKK2,** like RIP and NIK, are serine-threonine protein **kinases** involved in the activation of NF-.kappa.B pathway. The co-immunoprecipitation assay followed was similar to that described in Example 24 except the first expression vector consisted of myc-tagged Caspase 8 C360S, and the second expression vector consisted of FLAG-tagged **iKK1 or iKK2** respectively. FLAG-tagged proteins were immunoprecipitated using FLAG beads or control beads. Co-immunoprecipitating

myc-Caspase 8 was detected by western blot analysis using a rabbit polyclonal antibody against the myc tag.

Detailed Description Text - DETX:

The results of this experiment are shown in FIG. 40 and demonstrate that Caspase 8 directly interacts with **IKK1 and IKK2** proteins when co-expressed with **IKK1 or IKK2** in mammalian cells. Furthermore, combined with Examples 29, 31, and 33, this example confirms that Caspases and DEDs-containing proteins can interact with serine-threonine protein **kinases**. An assay based on interaction between Caspases and DEDs-containing proteins with **IKK1 or IKK2** can be used as a screening tool for identifying lead compounds for pharmacological agents capable of modulating interactions between Caspases (and/or DEDs-containing proteins) and **IKK1 or IKK2** for the purpose of diagnosis and treatment of diseases associated with the dysfunction of DEDs-containing proteins-IKK1/IKK2 and Caspases-IKK1/IKK2 signal transduction pathways. Similar experiments confirmed that **IKK1** also interacts with MRIT-.alpha.1.

US-PAT-NO: 6030834

DOCUMENT-IDENTIFIER: US 6030834 A

TITLE: **Human IKK**-beta DNA constructs and cells

DATE-ISSUED: February 29, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chu; Keting	Burlingame	CA	N/A	N/A
Pot; David	San Francisco	CA	N/A	N/A

APPL-NO: 09/ 215131

DATE FILED: December 18, 1998

PARENT-CASE:

This application claims the benefit of provisional application Ser. No. 60/068,954 filed Dec. 30, 1997, which is incorporated herein by reference.

US-CL-CURRENT: 435/325; 435/194 ; 435/253.2 ; 435/254.2 ; 435/320.1 ; 435/455 ; 435/471 ; 536/23.1 ; 536/23.2 ; 536/23.5

ABSTRACT:

A novel kinase has been identified which phosphorylates I.kappa.B. Reagents which inhibit this kinase can be used as therapeutic tools to inhibit inflammation. The kinase can also be used as a target for drug screening to identify anti-inflammatory compounds.

15 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

TITLE - TI:

Human IKK-beta DNA constructs and cells

Brief Summary Text - BSTX:

The diverse initial activation factors of NF.kappa.B converge into the final step of activation of I.kappa.B **kinase (IKK)**, which specifically phosphorylates

the inhibitory I.kappa.B factor and results in subsequent degradation of I.kappa.B factors. **IKK** proteins are therefore critical targets for anti-inflammatory drug development. Thus, there is a need in the art for identifying new components of this important regulatory system.

Other Reference Publication - OREF:

Mercurio F. et al. "**IKK-1 and IKK**-2: cytokine-activated IkappaB **kinases** essential for NF -kappaB activation" Science, vol. 278, Oct. 31, 1997 pp. 860-866.

US-PAT-NO: 5972655

DOCUMENT-IDENTIFIER: US 5972655 A

TITLE: Y2H61 an IKK binding protein

DATE-ISSUED: October 26, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Marcu; Kenneth B.	Stony Brook	NY	N/A	N/A

APPL-NO: 09/ 196048

DATE FILED: November 19, 1998

US-CL-CURRENT: 435/69.1; 435/252.3 ; 435/320.1 ; 435/325

ABSTRACT:

The present invention provides an isolated I.kappa.B kinase binding protein designated Y2H61 and functional equivalents therof. The amino acid sequence of Y2H61, the nucleotide sequence encoding Y2H61, and other related protein and nucleic acid molecules are also provided.

5 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Brief Summary Text - BSTX:

NF-.kappa.B is anchored in the cytoplasm of most non-stimulated cells by a non-covalent interaction with one of several inhibitory proteins known as I.kappa.Bs. See for example, Baeuerle and Baltimore, Science 242, 540-546 (1988). Cellular stimuli associated with immune and inflammatory responses, for example inflammatory cytokines such as tumor necrosis factor .alpha.(TNF.alpha.) or interleukin-1 (IL-1), activate **kinases,** which in turn activate NF-.kappa.B by phosphorylating I.kappa.Bs. The **kinases** that phosphorylate I.kappa.Bs are called I.kappa.B **kinases (IKKs**).

Brief Summary Text - BSTX:

IKK.alpha. and IKK.beta. have structural motifs characteristic of the **IKK kinases**. This includes an amino terminal serine-threonine **kinase** domain

separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper domain. These structural characteristics are unlike other **kinases,** and the domains are thought to be involved in protein-protein interactions.

Brief Summary Text - BSTX:

The term **IKK** is used herein to refer to all **kinases** that phosphorylate any I.kappa.B and that have helix-loop-helix and leucine zipper domains. Y2H61 and its functional equivalents bind to the region of the **IKK** proteins made up of the contiguous helix-loop-helix and leucine zipper domains.

Detailed Description Text - DETX:

A yeast two hybrid screen was undertaken with **IKKA** as a bait in an attempt to identify interacting proteins which could represent in vivo regulators of the cytokine induced **kinase** cascade. Full length IKK.alpha. and smaller fragments in the Field's pGTB9c bait vector (see Fields and Sternglanz, Trends Genet. 10, 286-292 (1994)) met with technical problems owing to its inherent in vivo transactivation properties. These problems were overcome by incorporating a high dose of an inhibitor of the product of the His3 selection gene thereby severely restricting yeast colony growth. (Triazole or 3-AT (3-amino-1, 2, 4-triazole or aminotriazole) has been reported to competitively inhibit the product of the yeast His3 gene in a dose dependent manner (Klopotowski et al., Arch. Biochem. Biophys. 112, 562-566 (1965)). The bait vector's insert was 937 bp SnaB1/XhoI fragment of the **murine** IKK.alpha. clone encoding the protein's leucine zipper, helix-loop-helix and carboxyl terminus. The latter bait vector was transfected into the Y153 yeast strain and a colony that grew on agar without tryptophan was selected for further transfections according to standard protocols (Yeast Matchmaker manual, Clontech Inc., Palo Alto, Calif.). Yeast harboring the bait grew on histidine-minus plates. However, this non-specific growth was abrogated by the inclusion of 50 .mu.M (3-AT) that would also yield the strongest interactors. Y153 cells harboring the bait vector were transfected with a B lymphoblast cDNA library (0.6.times.10.sup.9 cfu, ATCC #87003) (Durfee et al., Genes Dev 7, 555-569 (1993)) sub-cloned into plasmid BNN132 (for a final transfection frequency of 10.sup.5 clones), which were spread onto 30 agar plates (His-, Trp-, Leu-, 50 mM 3-AT). 126 clones showing a faster growth rate compared to background colonies were picked after 3 and 6 days incubation at 30.degree. C. and replated. 70 clones were selected for plasmid isolation based on their growth on His-, Trp-, Leu-, 50 .mu.M 3-AT plates. From these 70 picks secondary picks, 16 clones remained positive after multiple rounds of purification and rescreening (14 of these sixteen were unique and two were isolated twice).

Other Reference Publication - OREF:

Zandi et al., "The I.kappa.B Kinase Complex (IKK) Contains Two Kinase Subunits, IKK.alpha. and IKK.beta., Necessary for I.kappa.B Phosphorylation and NF-.kappa.B Activation", Cell 91:243-252 (1997).

US-PAT-NO: 5939302

DOCUMENT-IDENTIFIER: US 5939302 A

TITLE: IKK-.beta. proteins, nucleic acids and methods

DATE-ISSUED: August 17, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goeddel; David V.	Hillsborough	CA	N/A	N/A
Woronicz; John	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 099124

DATE FILED: June 17, 1998

PARENT-CASE:

INTRODUCTION This is a divisional application of U.S. Ser. No. 08/890,853, filed Jul. 10, 1997, which is a continuing application under 35USC120 of U.S. Ser. No. 08/887,114 filed Jul. 1, 1997, abandoned, both of which are incorporated herein by reference.

US-CL-CURRENT: 435/194

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

6 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **kinase** I.kappa.B **Kinase, IKK**-.beta., as a NIK-interacting protein. **IKK**-.beta. has sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.beta. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.beta. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.beta. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.beta. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.beta.-specific structure and activity and modulators of **IKK**-.beta. function, particularly I.kappa.B **kinase** activity. **IKK**-.beta. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.beta. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. gene, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.beta. transcripts), therapy (e.g. **IKK**-.beta. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.beta. polypeptide is shown as SEQ ID NO:1, and the full conceptual translate is shown as SEQ ID NO:2. The **IKK**-.beta. polypeptides of the invention include incomplete translates of SEQ ID NO: 1 and deletion mutants of SEQ ID NO:2, which translates and deletion mutants have **IKK**-.beta.-specific amino acid sequence and binding specificity or function.

Brief Summary Text - BSTX:

The **IKK**-.beta. polypeptide domains of the invention have amino acid sequences distinguishable from **IKK**-.alpha., generally at least 11, preferably at least 21, more preferably at least 31 consecutive residues of SEQ ID NO:2 and provide **IKK**-.beta. domain specific activity or function, such as **IKK**-.beta.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)).

Brief Summary Text - BSTX:

IKK-.beta.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.beta. **p**olypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.beta. substrate, a IKK-.beta. regulating protein or other regulator that directly modulates IKK-.beta. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.beta. specific agent such as those identified in screening assays such as described below. IKK-.beta.**-b**inding specificity may assayed by kinase ac**tivity** or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.beta.**-e**xpressing cells, to elicit IKK-.beta. specific antibody in a heterologous host (e.g. a rodent or rabbit), etc. In any event, the IKK-.beta. binding specificity of the subject IKK-.beta. polypeptides necessarily distinguishes IKK-.alph**a.** (SEQ ID NO: 4).

Brief Summary Text - BSTX:

The claimed **IKK**-.beta. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The **IKK**-.beta. polypeptides and polypeptide domains may be

synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to the subject **kinase** proteins including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-.beta.-specific binding agents include **IKK**-.beta.-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK**-.beta. function, e.g. **IKK**-.beta.-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK**-.beta. I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK**-.beta. I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-.beta.-derived peptide inhibitors, etc., see Tables 1 and 2. **IKK**-.beta. specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) 1995 Jul;48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry D W et al. Science 1994 Aug 19;265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science 1995 Mar 24;267(5205): 1782-8). Additional **IKK**-.beta. inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz A F et al., Proc Natl Acad Sci USA 1995 Feb 28;92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell 1995 Dec 15;83(6):1001-9).

Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby 0, et al. Proc Natl Acad Sci USA 1994 May 24;91(11):4761-5; Barja P, et al., Cell Immunol 1994 Jan;153(1):28-38; Villar-Palasi C, Biochim Biophys Acta 1994 Dec 30;1224(3):384-8; Liu W Z, et al., Biochemistry 1994 Aug 23;33(33):10120-6).

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK**-.beta. **kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK**-.beta. binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK**-.beta. modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK**-.beta. interaction with a natural **IKK**-.beta. binding target, in particular, **IKK**-.beta. phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Brief Summary Text - BSTX:

In vitro binding assays employ a mixture of components including an **IKK**-.beta. polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK**-.beta. binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta. or .epsilon.-derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so

long as the portion provides binding affinity and avidity to the subject **IKK**-.beta. polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK**-.beta. polypeptide and one or more binding targets is detected by any convenient way. For **IKK**-.beta. **kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.beta. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Text - BSTX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we initially identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic a-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Brief Summary Text - BSTX:

To identify potential **IKK**-.alpha.-related genes, we searched a public database of **human** expressed sequence tags (ESTs). We identified two ESTs (W68756 and

AA128064), which we determined were capable of encoding distinct peptides with sequence similarity with **IKK**-.alpha.. **IKK**-.alpha.-related cDNA was cloned by probing a Jurkat cDNA library (**human** T cell) with an oligonucleotide probe corresponding to sequence from one of the ESTs. Sequence analysis demonstrated that the two ESTs included different fragments of the same gene. The longest cDNA clones obtained had a .about.3.2 kb insert (SEQ ID NO: 1) and an open reading frame of 756 amino acids (SEQ ID NO: 2). We have designed the protein encoded by this cDNA as **IKK**-.beta..

Brief Summary Text - BSTX:

Interaction of **IKK**-.beta. and NIK in **Human** Cells

Brief Summary Text - BSTX:

The interaction of **IKK**-.beta. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.beta. containing an C-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK was detected by immunoblot analysis with an anti-Myc monoclonal antibody. In this assay, **IKK**-.beta. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.beta. protein lacking most of the N-terminal **kinase** domain (**IKK**-.beta..sub.(.DELTA.5-203, i.e. 1-4 & 204-756)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.beta. mediates the interaction with NIK.

Brief Summary Text - BSTX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.beta..sub.(.DELTA.5-203) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.beta..sub.(.DELTA.5-203) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.beta..sub.(.DELTA.5-203) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.beta. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.beta. functions as a common mediator of NF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Brief Summary Paragraph Table - BSTL:

TABLE I _____ Selected Small Molecule **IKK**-.beta. **Kinase** Inhibitors Inhibitors Citations

_____ HA-100.sup.1 1. Hagiwara, M,. et al.

Mol. Pharmacol. 32:7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172:993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151:542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135:397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55:1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176:288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159:548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24:497 (1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8:732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39:1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238:1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167:657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525:198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255:1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265:5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142:436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261:989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29:577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23:5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37:482 (1990)

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Peptidyl **IKK**-.beta. **Kinase** Inhibitors hI.kappa.B.alpha., residues 24-39, 32Ala hIKK-.beta., .DELTA.5-203 hI.kappa.B.alpha., residues 29-47, 36Ala hIKK-.beta., .DELTA.1-178 hI.kappa.B.alpha., residues 26-46, 32/36Ala hIKK-.beta., .DELTA.368-756 hI.kappa.B.beta., residues 25-38, 32Ala hIKK-.beta., .DELTA.460-748 hI.kappa.B.beta., residues 30-41, 36Ala hIKK-.alpha., .DELTA.1-289 hI.kappa.B.beta., residues 26-46, 32/36Ala hIKK-.alpha., .DELTA.12-219 hI.kappa.B.epsilon., residues 24-40, 32Ala hIKK-.alpha., .DELTA.307-745 hI.kappa.B.epsilon., residues 31-50, 36Ala hIKK-.alpha., .DELTA.319-644 hI.kappa.B.epsilon., residues 27-44, 32/36Ala

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.beta._(SEQ ID NO: 2) at 20 .mu.g/ml in PBS.

US-PAT-NO: 5916760

DOCUMENT-IDENTIFIER: US 5916760 A

TITLE: IKK-.beta. proteins, nucleic acids and methods

DATE-ISSUED: June 29, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goeddel; David V.	Hillsborough	CA	N/A	N/A
Woronicz; John	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 099125

DATE FILED: June 17, 1998

PARENT-CASE:

This is a divisional application of U.S. Ser. No. 08/890,853, filed Jul. 10, 1997, now U.S. Pat. No. 5,851,812, which is a continuing application under 35USC120 of U.S. Ser. No. 08/887,114 filed Jul. 1, 1997, abandoned, both of which are incorporated herein by reference.

US-CL-CURRENT: 435/15; 435/194

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

16 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **kinase** I.kappa.B **Kinase, IKK**-.beta., as a NIK-interacting protein. **IKK**-.beta. has sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.beta. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.beta. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.beta. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSMIL (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I-.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.beta. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.beta.-specific structure and activity and modulators of **IKK**-.beta. function, particularly I.kappa.B **kinase** activity. **IKK**-.beta. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.beta. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. gene, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.beta. transcripts), therapy (e.g. **IKK**-.beta. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Detailed Description Text - DETX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.beta. polypeptide is shown as SEQ ID NO: 1, and the full conceptual translate is shown as SEQ ID NO:2. The **IKK**-.beta. polypeptides of the invention include incomplete translates of SEQ ID NO: 1 and deletion mutants of SEQ ID NO:2, which translates and deletion mutants have **IKK**-.beta.-specific amino acid sequence and binding specificity or function.

Detailed Description Text - DETX:

The **IKK**-.beta. polypeptide domains of the invention have amino acid sequences distinguishable from **IKK**-.alpha., generally at least 11, preferably at least 21, more preferably at least 31 consecutive residues of SEQ ID NO:2 and provide **IKK**-.beta. domain specific activity or function, such as **IKK**-.beta.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)).

Detailed Description Text - DETX:

IKK-.beta.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.beta. **p**olypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.beta._substrate, a IKK-.beta._regulating protein or other regulator that directly modulates IKK-.beta._activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.beta._specific agent such as those identified in screening assays such as described below. IKK-.beta.-**b**inding specificity may assayed by kinase ac**tivity** or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.beta.-**e**xpressing cells, to elicit IKK-.beta._specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the IKK-.beta._binding specificity of the subject IKK-.beta._polypeptides necessarily distinguishes IKK-.alpha._ (SEQ ID NO:4).

Detailed Description Text - DETX:

The claimed **IKK**-.beta. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99%. by weight of the total polypeptide in a given sample. The **IKK**-.beta. polypeptides and polypeptide domains may be

synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Detailed Description Text - DETX:

The invention provides binding agents specific to the subject **kinase** proteins including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-.beta.-specific binding agents include **IKK**-.beta.-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK**-.beta. function, e.g. **IKK**-.beta.-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK**-.beta. I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK**-.beta. I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-.beta.-derived peptide inhibitors, etc., see Tables 1 and 2. **IKK**-.beta. specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Detailed Description Text - DETX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) 1995 Jul;48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry D. W. et al. Science 1994 Aug 19;265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science 1995 Mar 24;267(5205):1782-8). Additional **IKK**-.beta. inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz AF et al., Proc Natl Acad Sci USA 1995 Feb 28;92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell 1995 Dec 15;83(6):1001-9).

Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby 0, et al. Proc Natl Acad Sci USA 1994 May 24;91(11):4761-5; Barja P, et al., Cell Immunol 1994 Jan;153(1):28-38; Villar-Palasi C, Biochim Biophys Acta 1994 Dec 30;1224(3):384-8; Liu W. Z., et al., Biochemistry 1994 Aug 23;33(33):10120-6).

Detailed Description Text - DETX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK**-.beta. **kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK**-.beta. binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Detailed Description Text - DETX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK**-.beta. modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK**-.beta. interaction with a natural **IKK**-.beta. binding target, in particular, **IKK**-.beta. phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Detailed Description Text - DETX:

In vitro binding assays employ a mixture of components including an **IKK**-.beta. polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK**-.beta. binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha.,.beta. or .epsilon.-derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so

long as the portion provides binding affinity and avidity to the subject **IKK**-.beta. polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Detailed Description Text - DETX:

After incubation, the agent-biased binding between the **IKK**-.beta. polypeptide and one or more binding targets is detected by any convenient way. For **IKK**-.beta. **kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.beta. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Detailed Description Text - DETX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we initially identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK-a** two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic .alpha.-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Detailed Description Text - DETX:

To identify potential **IKK**-.alpha.-related genes, we searched a public database of **human** expressed sequence tags (ESTs). We identified two ESTs (W68756 and

AA128064), which we determined were capable of encoding distinct peptides with sequence similarity with **IKK**-.alpha.. **IKK**-.alpha.-related cDNA was cloned by probing a Jurkat cDNA library (**human** T cell) with an oligonucleotide probe corresponding to sequence from one of the ESTs. Sequence analysis demonstrated that the two ESTs included different fragments of the same gene. The longest cDNA clones obtained had a .about.3.2 kb insert (SEQ ID NO:1) and an open reading frame of 756 amino acids (SEQ ID NO:2). We have designed the protein encoded by this cDNA as **IKK**-.beta..

Detailed Description Text - DETX:

Interaction of **IKK**-.beta. and NIK in **Human** Cells

Detailed Description Text - DETX:

The interaction of **IKK**-.beta. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.beta. containing an C-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK was detected by immunoblot analysis with an anti-Myc monoclonal antibody. In this assay, **IKK**-.beta. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.beta. protein lacking most of the N-terminal **kinase** domain (**IKK**-.beta..sub.(.DELTA.5-203, i.e., 1-4 & 204-756)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.beta. mediates the interaction with NIK.

Detailed Description Text - DETX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.beta..sub.(.DELTA.5-203) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.beta..sub.(.DELTA.5-203) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.beta..sub.(.DELTA.5-203) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.beta. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.beta. functions as a common mediator of KF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.beta._(SEQ ID NO:2) at 20 .mu.g/ml in PBS.

Detailed Description Paragraph Table - DETL:

TABLE I _____ Selected Small Molecule **IKK**-.beta. **Kinase** Inhibitors Inhibitors Citations _____ HA-100.sup.1 1. Hagiwara, M,. et al. Mol. Pharmacol. 32: 7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172: 993 (1990) Staurosporine.sup.3,4,5 3. Schachtele, C., et al. Biochem Biophys Res Com 151: 542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135: 397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55: 1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176: 288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159: 548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24: 497 (1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8: 732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39: 1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238: 1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167: 657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525: 198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255: 1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265: 5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142: 436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261: 989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29: 577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231: 141 (1984) 20. Hidaka, H., et al. Biochemistry 23: 5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143: 1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37: 482 (1990) _____

Detailed Description Paragraph Table - DETL:

TABLE II _____ Selected Peptidyl **IKK**-.beta. **Kinase** Inhibitors _____ hI.kappa.B.alpha., residues 24-39, 32Ala hIKK-.beta., .DELTA.5-203 hI.kappa.B.alpha., residues 29-47, 36Ala hIKK-.beta., .DELTA.1-178 hI.kappa.B.alpha., residues 26-46, 32/36Ala hIKK-.beta., .DELTA.368-756 hI.kappa.B.beta., residues 25-38, 32Ala hIKK-.beta., .DELTA.460-748 hI.kappa.B.beta., residues 30-41, 36Ala hIKK-.alpha., .DELTA.1-289 hI.kappa.B.beta., residues 26-46, 32/36Ala hIKK-.alpha., .DELTA.12-219 hI.kappa.B.epsilon., residues 24-40, 32Ala hIKK-.alpha., .DELTA.307-745 hI.kappa.B.epsilon., residues 31-50, 36Ala hIKK-.alpha., .DELTA.319-644 hI.kappa.B.epsilon., residues 27-44, 32/36Ala

_____

US-PAT-NO: 5891719

DOCUMENT-IDENTIFIER: US 5891719 A

TITLE: IKAP nucleic acids

DATE-ISSUED: April 6, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Cohen; Lucie	South San Francisco	CA	N/A	N/A
Baeuerle; Patrick	South San Francisco	CA	N/A	N/A

APPL-NO: 08/ 971244

DATE FILED: November 16, 1997

US-CL-CURRENT: 435/325; 435/252.3 ; 435/254.11 ; 435/69.1 ; 530/300 ; 530/350 ; 536/23.1 ; 536/23.5 ; 536/24.3 ; 536/24.31

ABSTRACT:

The invention provides methods and compositions relating to IKAP proteins which regulate cellular signal transduction and transcriptional activation, and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed IKAP encoding nucleic acids or purified from human cells. The invention provides isolated IKAP hybridization probes and primers capable of specifically hybridizing with the disclosed IKAP genes, IKAP-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

46 Claims, 1 Drawing figures

Exemplary Claim Number: 24

Number of Drawing Sheets: 1

---------- KWIC ---------

Brief Summary Text - BSTX:

The NF-.kappa.B-inducing **kinase** (NIK) is a member of the MAP **kinase kinase kinase** (MAP3K) family that was identified as a TRAF2-interacting protein (Malinin et al., 1997). NIK activates NF-.kappa.B when overexpressed, and

kinase-inactive mutants of NIK comprising its TRAF2-interacting C-terminal domain (NIK.sub.(624-947)) or lacking two crucial lysine residues in its **kinase** domain (NIK.sub.(KK429-430AA)) behave as dominant-negative inhibitors that suppress TNF-, IL-1-, and TRAF2-induced NF-.kappa.B activation (Malinin et al., 1997). Recently, NIK was found to associate with additional members of the TRAF family, including TRAF5 and TRAF6. Catalytically inactive mutants of NIK also inhibited TRAF5- and TRAF6-induced NF-.kappa.B activation, thus providing a unifying concept for NIK as a common mediator in the NF-.kappa.B signaling cascades triggered by TNF and IL-1 downstream of TRAFs. Recently two NIK-interacting protein designated characterized as novel **human kinase** I.kappa.B **Kinases, IKK**-.alpha. and **IKK**-.beta. have been reported (Woronicz et al., 1997; Mercurio et al. 1997; Maniatis, 1997). Catalytically inactive mutants of **IKK** suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK** associates with endogenous I.kappa.B.alpha. complex; and **IKK** phosphorylates I.kappa.B.alpha. on serines 32 and 36.

US-PAT-NO: 5854003

DOCUMENT-IDENTIFIER: US 5854003 A

TITLE: Screening method for agents that modulate human NIK activity

DATE-ISSUED: December 29, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Wu; Lin	South San Francisco	CA	N/A	N/A

APPL-NO: / 032475

DATE FILED: February 26, 1998

PARENT-CASE:

This is a divisional application of U.S. Ser. No. 08/887,518, filed Jul. 3, 1997.

US-CL-CURRENT: 435/7.8

ABSTRACT:

The invention provides methods and compositions relating to a novel kinase, NIK, involved in NF.kappa.B activation. The polypeptides may be produced recombinantly from transformed host cells from the disclosed NIK encoding nucleic acids or purified from human cells. The invention provides isolated NIK hybridization probes and primers capable of specifically hybridizing with the disclosed NIK genes, NIK-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

9 Claims, 0 Drawing figures

Exemplary Claim Number: 1

--------- KWIC ---------

Brief Summary Text - BSTX:

Here, we disclose a novel **human** NIK (NIK.sub.(Ala25)), which also provides the foregoing functionalities yet deviates in terms of critical sequence and structural characteristics; in particular, a Pro-Ala substitution at position

25 imposes altered protein structure. We show that the NIK.sub.(Ala25) variant interacts with and cross-phosphorylates the I.kappa.B **Kinases** .alpha. and .beta., **IKK**-.alpha. and **IKK**-.beta. (see Goeddel et al. and Rothe et al., copending applications T97-006 and T97-007, respectively, filed Jul. 1, 1997). **IKK**-.alpha. and **IKK**-.beta. have sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of the **IKKs** suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKKs** associate with the endogenous I.kappa.B.alpha. complex; and the **IKKs** phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively. In addition, we disclose that NIK.sub.(Ala25) associates with other members of the TRAF family, including TRAF5 and TRAF6. Catalytically inactive mutants of NIK.sub.(Ala25) also inhibit TRAF5- and TRAF6-induced NF-.kappa.B activation, thus providing a unifying concept for NIK.sub.(Ala25) as a common mediator in the NF-.kappa.B signaling cascades triggered by TNF and IL-1 downstream of TRAFs.

Brief Summary Text - BSTX:

The nucleotide sequence of a natural **human** cDNA encoding a **human** NIK polypeptide is shown as SEQ ID NO:1, and the full conceptual translate is shown as SEQ ID NO:2. This novel NIK cDNA sequence was cloned by PCR using primers designed from GenBank accesion number Y102565. The NIK polypeptides of the invention include incomplete translates of SEQ ID NO:1 which translates and deletion mutants of SEQ ID NO:2 have **human** NIK-specific amino acid sequence, binding specificity or function and comprise Ala25. Preferred translates/deletion mutants comprise at least a 10 residue Ala25-containing domain of SEQ ID NO:2, preferably including residues 22-31, more preferably including residues 12-31, most preferably including residues 2-31. The subject domains provide NIK domain specific activity or function, such as NIK-specific **kinase or kinase** inhibitory activity, **IKK**-.alpha./.beta. (SEQ ID NO:3/4, respectively)-binding or binding inhibitory activity, TRAF1, 2, 3, 5 and/or 6 binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one serine residue of **IKK**-.alpha. and/or .beta..

Brief Summary Text - BSTX:

The invention provides binding agents specific to the claimed NIK polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic

applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel NIK-specific binding agents include NIK-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g. Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate NIK function, e.g. NIK-dependent transcriptional activation. For example, a wide variety of inhibitors of NIK **IKK**-.alpha./.beta. **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary NIK **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, NIK-derived peptide inhibitors, esp. dominant negative deletion mutants, etc., see Tables 1 and 2. NIK specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Claims Text - CLTX:

8. The method according to claim 7 wherein said substrate comprises an I.kappa.B **kinase (IKK**) polypeptide domain.

US-PAT-NO: 5851812

DOCUMENT-IDENTIFIER: US 5851812 A

TITLE: IKK-.beta. proteins, nucleic acids and methods

DATE-ISSUED: December 22, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goeddel; David V.	Hillsborough	CA	N/A	N/A
Woronicz; John	South San Francisco	CA	N/A	N/A

APPL-NO: 08/ 890853

DATE FILED: July 10, 1997

PARENT-CASE:

This a continuing application under 35USC120 of U.S. Ser. No. 08/887,114 filed Jul. 1, 1997, abandoned.

US-CL-CURRENT: 435/194; 435/252.3 ; 435/325 ; 536/23.2

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

12 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.beta., and related nucleic acids. The polypeptides may be

produced recombinantly from transformed host cells from the disclosed **IKK**-.beta. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. genes, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **kinase** I.kappa.B **Kinase, IKK**-.beta., as a NIK-interacting protein. **IKK**-.beta. has sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.beta. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.beta. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.beta. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.beta. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.beta.-specific structure and activity and modulators of **IKK**-.beta. function, particularly I.kappa.B **kinase** activity. **IKK**-.beta. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.beta. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.beta. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.beta. gene, **IKK**-.beta.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.beta. transcripts), therapy (e.g. **IKK**-.beta. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.beta. polypeptide is shown as SEQ ID NO: 1, and the full conceptual translate is

shown as SEQ ID NO: 2. The IKK-.beta. polypeptides of the invention include incomplete translates of SEQ ID NO: 1 and deletion mutants of SEQ ID NO: 2, which translates and deletion mutants have IKK-.beta.-specific amino acid sequence and binding specificity or function.

Brief Summary Text - BSTX:

The IKK-.beta. polypeptide domains of the invention have amino acid sequences distinguishable from IKK-.alpha., generally at least 11, preferably at least 21, more preferably at least 31 consecutive residues of SEQ ID NO: 2 and provide IKK-.beta. domain specific activity or function, such as IKK-.beta.-specific kinase or kinase inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)).

Brief Summary Text - BSTX:

IKK-.beta.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.beta. polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.beta. substrate, a IKK-.beta. regulating protein or other regulator that directly modulates IKK-.beta. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.beta. specific agent such as those identified in screening assays such as described below. IKK-.beta.-binding specificity may assayed by kinase activity or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.beta.-expressing cells, to elicit IKK-.beta. specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the IKK-.beta. binding specificity of the subject IKK-.beta. polypeptides necessarily distinguishes IKK-.alpha. (SEQ ID NO: 4).

Brief Summary Text - BSTX:

The claimed IKK-.beta. polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The IKK-.beta. polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods

are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to the subject **kinase** proteins including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-.beta.-specific binding agents include **IKK**-.beta.-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK**-.beta. function, e.g. **IKK**-.beta.-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK**-.beta. I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK**-.beta. I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-.beta.-derived peptide inhibitors, etc., see Tables 1 and 2. **IKK**-.beta. specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) 1995 July;48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry D W et al. Science 1994 Aug. 19;265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science 1995 Mar 24;267(5205):1782-8). Additional **IKK**-.beta. inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz A F et al., Proc Natl Acad Sci USA 1995 Feb. 28;92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell 1995 Dec. 15;83(6):1001-9). Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate

competition) are readily generated by established methods (Hvalby O, et al. Proc Natl Acad Sci USA 1994 May 24;91(11):4761-5; Barja P, et al., Cell Immunol 1994 January;153(1):28-38; Villar-Palasi C, Biochim Biophys Acta 1994 Dec. 30;1224(3):384-8; Liu W Z, et al., Biochemistry 1994 Aug. 23;33(33):10120-6).

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK**-.beta. **kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK**-.beta. binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK**-.beta. modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK**-.beta. interaction with a natural **IKK**-.beta. binding target, in particular, **IKK**-.beta. phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Brief Summary Text - BSTX:

In vitro binding assays employ a mixture of components including an **IKK**-.beta. polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular **IKK**-.beta. binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32 and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta. or .epsilon. -derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject **iKK**-.beta. polypeptide conveniently measurable in the assay. The assay

mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK**-.beta. polypeptide and one or more binding targets is detected by any convenient way. For **IKK**-.beta. **kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.beta. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Text - BSTX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we initially identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic .alpha.-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Brief Summary Text - BSTX:

To identify potential **IKK**-.alpha.-related genes, we searched a public database of **human** expressed sequence tags (ESTs). We identified two ESTs (W68756 and AA128064), which we determined were capable of encoding distinct peptides with sequence similarity with **IKK**-.alpha.. **IKK**-.alpha.-related cDNA was cloned by

probing a Jurkat cDNA library (**human** T cell) with an oligonucleotide probe corresponding to sequence from one of the ESTs. Sequence analysis demonstrated that the two ESTs included different fragments of the same gene. The longest cDNA clones obtained had a .about.3.2 kb insert (SEQ ID NO: 1) and an open reading frame of 756 amino acids (SEQ ID NO: 2). We have designed the protein encoded by this cDNA as **IKK**-.beta..

Brief Summary Text - BSTX:

Interaction of **IKK**-.beta. and NIK in **Human** Cells

Brief Summary Text - BSTX:

The interaction of **IKK**-.beta. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.beta. containing an C-terminal Flag epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK was detected by immunoblot analysis with an anti-Myc monoclonal antibody. In this assay, **IKK**-.beta. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.beta. protein lacking most of the N-terminal **kinase** domain (**IKK**-.beta..sub.(.DELTA.5-203, i.e. 1-4 & 204-756)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.beta. mediates the interaction with NIK.

Brief Summary Text - BSTX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.beta..sub.(.DELTA.5-203) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.beta..sub.(.DELTA.5-203) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.beta..sub.(.DELTA.5-203) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.beta. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.beta. functions as a common mediator of NF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Brief Summary Paragraph Table - BSTL:

TABLE I _____ Selected Small Molecule **IKK**-.beta. **Kinase** Inhibitors Inhibitors Citations

_____ HA-100.sup.1 1. Hagiwara, M,. et al. Mol. Pharmacol. 32:7 (1987) Chelerythrine.sup.2 2. Herbert, J. M., et al. Biochem Biophys Res Com 172:993 (1990) Staurosporine.sup.3,4,5 3.

Schachtele, C., et al. Biochem Biophys Res Com 151:542 (1988) Calphostin C.sup.6,7,8,9 4. Tamaoki, T., et al. Biochem Biophys Res Com 135:397 (1986) K-252b.sup.10 5. Tischler, A. S., et al. J. Neurochemistry 55:1159 (1990) PKC 19-36.sup.11 6. Bruns, R. F., et al. Biochem Biophys Res Com 176:288 (1991) Iso-H7.sup.12 7. Kobayashi, E., et al. Biochem Biophys Res Com 159:548 (1989) PKC 19-31 8. Tamaoki, T., et al Adv 2nd Mass Phospho- protein Res 24:497 (1990) H-7.sup.13,3,14 9. Tamaoki, T., et al. Biotechnology 8:732 (1990) H-89.sup.15 10. Yasuzawa, T. J. Antibiotics 39:1972 (1986) KT5720.sup.16 11. House, C., et al. Science 238:1726 (1987) cAMP-depPKinhib.sup.17 12. Quick, J., et al. Biochem. Biophys. Res. Com. 167:657 (1992) A-3.sup.18 13. Bouli, N. M. and Davis, M. Brain Res. 525:198 (1990) HA1004.sup.19,20 14. Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255:1218 (1990) K-252a.sup.16,5 15. Chijiwa, T., et al. J. Biol. Chem. 265:5267 (1990) KT5823.sup.16 16. Kase, H., et al. Biochem. Biophys. Res. Com. 142:436 (1987) ML-9.sup.21 17. Cheng, H. C., et al. J. Biol. Chem. 261:989 (1986) KT5926.sup.22 18. Inagaki, M., et al. Mol. Pharmacol. 29:577 (1986) 19. Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231:141 (1984) 20. Hidaka, H., et al. Biochemistry 23:5036 (1984) 21. Nagatsu, T., et al. Biochem Biophys Res Com 143:1045 (1987) 22. Nakanishi, S., et al. Mol. Pharmacol. 37:482 (1990)

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Peptidyl **IKK**-.beta. **Kinase** Inhibitors hI.kappa.B.alpha., residues 24-39, 32Ala hIKK-.beta., .DELTA.5-203 hI.kappa.B.alpha., residues 29-47, 36Ala hIKK-.beta., .DELTA.1-178 hI.kappa.B.alpha., residues 26-46, 32/36Ala hIKK-.beta., .DELTA.368-756 hI.kappa.B.beta., residues 25-38, 32Ala hIKK-.beta., .DELTA.460-748 hI.kappa.B.beta., residues 30-41, 36Ala hIKK-.alpha., .DELTA.1-289 hI.kappa.B.beta., residues 26-46, 32/36Ala hIKK-.alpha., .DELTA.12-219 hI.kappa.B.epsilon., residues 24-40, 32Ala hIKK-.alpha., .DELTA.307-745 hI.kappa.B.epsilon., residues 31-50, 36Ala hIKK-.alpha., .DELTA.319-644 hI.kappa.B.epsilon., residues 27-44, 32/36Ala

Detailed Description Text - DETX:

kinase: 10.sup.-8 -10.sup.-5 M IKK-.beta. (SEQ ID NO: 2) at 20 .mu.g/ml in PBS.

US-PAT-NO: 5844073

DOCUMENT-IDENTIFIER: US 5844073 A

TITLE: Human NIK proteins

DATE-ISSUED: December 1, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Wu; Lin	South San Francisco	CA	N/A	N/A

APPL-NO: / 023321

DATE FILED: February 13, 1998

PARENT-CASE:

This is a divisional application of U.S. Ser. No. 08/887,518, filed Jul. 3, 1997.

US-CL-CURRENT: 530/300; 530/324 ; 530/325 ; 530/326 ; 530/327 ; 530/328 ; 530/350

ABSTRACT:

The invention provides methods and compositions relating to a novel kinase, NIK, involved in NF.kappa.B activation. The polypeptides may be produced recombinantly from transformed host cells from the disclosed NIK encoding nucleic acids or purified from human cells. The invention provides isolated NIK hybridization probes and primers capable of specifically hybridizing with the disclosed NIK genes, NIK-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

6 Claims, 0 Drawing figures

Exemplary Claim Number: 2

---------- KWIC ---------

Brief Summary Text - BSTX:

Here, we disclose a novel **human** NIK (NIK .sub.(Ala25)), which also provides the foregoing functionalities yet deviates in terms of critical sequence and

structural characteristics; in particular, a Pro-Ala substitution at position 25 imposes altered protein structure. We show that the NIK .sub.(Ala25) variant interacts with and cross-phosphorylates the I.kappa.B **Kinases** .alpha. and .beta., **IKK**-.alpha. and **IKK**-.beta. (see Goeddel et al. and Rothe et al., copending applications T97-006 (U.S. Ser. No. 08/887,114) and T97-007 (U.S. Ser. No. 08/887,115, abandoned), respectively, filed Jul. 1, 1997). **IKK**-.alpha. and **IKK**-.beta. have sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of the **IKKs** suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKKs** associate with the endogenous I.kappa.B.alpha. complex; and the **IKKs** phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively. In addition, we disclose that NIK .sub.(Ala25) associates with other members of the TRAF family, including TRAF5 and TRAF6. Catalytically inactive mutants of NIK .sub.(Ala25) also inhibit TRAF5- and TRAF6-induced NF-.kappa.B activation, thus providing a unifying concept for NIK .sub.(Ala25) as a common mediator in the NF-.kappa.B signaling cascades triggered by TNF and IL-1 downstream of TRAFs.

Brief Summary Text - BSTX:

The nucleotide sequence of a natural **human** cDNA encoding a **human** NIK polypeptide is shown as SEQ ID NO:1, and the full conceptual translate is shown as SEQ ID NO:2. This novel NIK cDNA sequence was cloned by PCR using primers designed from GenBank accesion number Y102565. The NIK polypeptides of the invention include incomplete translates of SEQ ID NO:1 which translates and deletion mutants of SEQ ID NO:2 have **human** NIK-specific amino acid sequence, binding specificity or function and comprise Ala25. Preferred translates/deletion mutants comprise at least a 10 residue Ala25-containing domain of SEQ ID NO:2, preferably including residues 22-31, more preferably including residues 12-31, most preferably including residues 2-31. The subject domains provide NIK domain specific activity or function, such as NIK-specific **kinase or kinase** inhibitory activity, **IKK**-.alpha./.beta. (SEQ ID NO:3/4, respectively)-binding or binding inhibitory activity, TRAF1, 2, 3, 5 and/or 6 binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one serine residue of **IKK**-.alpha. and/or .beta..

Brief Summary Text - BSTX:

The invention provides binding agents specific to the claimed NIK polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in

diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel NIK-specific binding agents include NIK-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate NIK function, e.g. NIK-dependent transcriptional activation. For example, a wide variety of inhibitors of NIK **IKK**-.alpha./.beta. **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary NIK **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, NIK-derived peptide inhibitors, esp. dominant negative deletion mutants, etc., see Tables 1 and 2. NIK specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

US-PAT-NO: 5843721

DOCUMENT-IDENTIFIER: US 5843721 A

TITLE: Nucleic acids encoding human NIK protein

DATE-ISSUED: December 1, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Wu; Lin	South San Francisco	CA	N/A	N/A

APPL-NO: 08/ 887518

DATE FILED: July 3, 1997

US-CL-CURRENT: 435/69.2; 435/243 ; 435/320.1 ; 435/325 ; 435/410 ; 435/69.1 ; 536/23.1 ; 536/23.5 ; 536/24.31

ABSTRACT:

The invention provides methods and compositions relating to a novel kinase, NIK, involved in NF.kappa.B activation. The polypeptides may be produced recombinantly from transformed host cells from the disclosed NIK encoding nucleic acids or purified from human cells. The invention provides isolated NIK hybridization probes and primers capable of specifically hybridizing with the disclosed NIK genes, NIK-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

15 Claims, 0 Drawing figures

Exemplary Claim Number: 3

---------- KWIC ---------

Brief Summary Text - BSTX:

Here, we disclose a novel **human** NIK(NIK .sub.(Ala25), which also provides the foregoing functionalities yet deviates in terms of critical sequence and structural characteristics; in particular, a Pro-Ala substitution at position 25 imposes altered protein structure. We show that the NIK.sub.(Ala25) variant interacts with and cross-phosphorylates the I.kappa.B **Kinases** .alpha. and .beta., **IKK**-.alpha. and **IKK**-.beta. (see Goeddel et al. and Rothe et al., copending applications T97-006 and T97-007, respectively, filed Jul. 1, 1997).

IKK-.alpha. and **IKK**-.beta.` have sequence similarity to the conceptual translate of a previously identified open reading frame postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of the **IKKs** suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKKs** associate with the endogenous I.kappa.B.alpha. complex; and the **IKKs** phosphorylate I.kappa.B.alpha. on serines 32 and 36. As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively. In addition, we disclose that NIK.sub.(Ala25) associates with other members of the TRAF family, including TRAF5 and TRAF6. Catalytically inactive mutants of NIK.sub.(Ala25) also inhibit TRAF5- and TRAF6-induced NF-.kappa.B activation, thus providing a unifying concept for NIK.sub.(Ala25) as a common mediator in the NF-.kappa.B signaling cascades triggered by TNF and IL-1 downstream of TRAFs.

Brief Summary Text - BSTX:

The nucleotide sequence of a natural **human** cDNA encoding a **human** NIK polypeptide is shown as SEQ ID NO: 1, and the fall conceptual translate is shown as SEQ ID NO:2. This novel NIK cDNA sequence was cloned by PCR using primers designed from GenBank accesion number Y102565. The NIK polypeptides of the invention include incomplete translates of SEQ ID NO: 1 which translates and deletion mutants of SEQ ID NO:2 have **human** NIK-specific amino acid sequence, binding specificity or function and comprise Ala25. Preferred translates/deletion mutants comprise at least a 10 residue Ala25-containing domain of SEQ ID NO:2, preferably including residues 22-31, more preferably including residues 12-31, most preferably including residues 2-31. The subject domains provide NIK domain specific activity or function, such as NIK-specific **kinase or kinase** inhibitory activity, **IKK**-.alpha./.beta. (SEQ ID NO:3/4, respectively)-binding or binding inhibitory activity, TRAF1, 2, 3, 5 and/or 6 binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one serine residue of **IKK**-.alpha. and/or .beta..

Brief Summary Text - BSTX:

The invention provides binding agents specific to the claimed NIK polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel NIK-specific binding agents include NIK-specific receptors, such as somatically recombined polypeptide receptors like specific

antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate NIK function, e.g. NIK-dependent transcriptional activation. For example, a wide variety of inhibitors of NIK **IKK**-.alpha./.beta. **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary NIK **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, NIK-derived peptide inhibitors, esp. dominant negative deletion mutants, etc., see Tables 1 and 2. NIK specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

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                  * * * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 15:35:08 ON 17 JUL 2002

=> fil .bec
COST IN U.S. DOLLARS                                          SINCE FILE      TOTAL
                                                                   ENTRY    SESSION
FULL ESTIMATED COST                                                0.21       0.21

FILES 'MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, HCAPLUS, NTIS,
       ESBIOBASE, BIOTECHNO, WPIDS' ENTERED AT 15:35:19 ON 17 JUL 2002
ALL COPYRIGHTS AND RESTRICTIONS APPLY.  SEE HELP USAGETERMS FOR DETAILS.

11 FILES IN THE FILE LIST

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L1          1537 IKK?

FILE 'SCISEARCH'
L2           637 IKK?

FILE 'LIFESCI'
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FILE 'BIOTECHDS'
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FILE 'BIOSIS'
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FILE 'HCAPLUS'
L7           580 IKK?

FILE 'NTIS'
L8            30 IKK?

FILE 'ESBIOBASE'
L9           348 IKK?

FILE 'BIOTECHNO'
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          99149 MURINE
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         239783 MOUSE
          99849 MURINE
L14           47 L2 (8A)(HUMAN OR MOUSE OR MURINE)
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         43097 HUMAN
         19954 MOUSE
          1440 MURINE
L16           6 L4  (8A)(HUMAN OR MOUSE OR MURINE)

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       5205997 HUMAN
        630504 MOUSE
        129349 MURINE
L17          68 L5  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'EMBASE'
       4442880 HUMAN
        491008 MOUSE
         88777 MURINE
L18          33 L6  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'HCAPLUS'
       1019282 HUMAN
        262670 MOUSE
         88267 MURINE
L19          78 L7  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'NTIS'
         78723 HUMAN
          3781 MOUSE
           868 MURINE
L20           0 L8  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'ESBIOBASE'
        312366 HUMAN
         73982 MOUSE
         33180 MURINE
L21          31 L9  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'BIOTECHNO'
        644107 HUMAN
        198561 MOUSE
         50977 MURINE
L22          27 L10(8A)(HUMAN OR MOUSE OR MURINE)

FILE 'WPIDS'
        106978 HUMAN
         16362 MOUSE
          2509 MURINE
L23           6 L11(8A)(HUMAN OR MOUSE OR MURINE)

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=> s l24 not 2000-2002/py
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       1223648 2000-2002/PY
L25          18 L13 NOT 2000-2002/PY

FILE 'SCISEARCH'
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        2374712 2000-2002/PY
L26           19 L14 NOT 2000-2002/PY

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         230210 2000-2002/PY
L27           16 L15 NOT 2000-2002/PY

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          32301 2000-2002/PY
L28            4 L16 NOT 2000-2002/PY

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L29           18 L17 NOT 2000-2002/PY

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        1068809 2000-2002/PY
L30           17 L18 NOT 2000-2002/PY

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L31           21 L19 NOT 2000-2002/PY

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L32           0 L20 NOT 2000-2002/PY

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         688659 2000-2002/PY
L33           16 L21 NOT 2000-2002/PY

FILE 'BIOTECHNO'
         290105 2000-2002/PY
L34           15 L22 NOT 2000-2002/PY

FILE 'WPIDS'
        2126954 2000-2002/PY
L35            2 L23 NOT 2000-2002/PY

TOTAL FOR ALL FILES
L36          146 L24 NOT 2000-2002/PY

=> dup rem l36
PROCESSING COMPLETED FOR L36
L37           35 DUP REM L36 (111 DUPLICATES REMOVED)

=> d tot

L37  ANSWER 1 OF 35  HCAPLUS  COPYRIGHT 2002 ACS        DUPLICATE 1
TI   Protein and cDNA sequences encoding human Y2H61, an I.kappa.B
     kinase-binding protein
SO   U.S., 8 pp.
     CODEN: USXXAM
IN   Marcu, Kenneth B.
AN   1999:686620  HCAPLUS
DN   131:318590
     PATENT NO.         KIND  DATE           APPLICATION NO.   DATE
     ---------------    ----  --------       ---------------   --------
PI   US 5972655          A    19991026       US 1998-196048    19981119

L37   ANSWER 2 OF 35  BIOTECHDS COPYRIGHT 2002 THOMSON DERWENT AND ISI
TI    Newly isolated human kinase, IkappaB-kinase (IKK
      -alpha) polypeptides;
         expression in host cell, DNA probe, DNA primer and antibody, used for
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diagnosis and therapy
AU Rothe M; Cao Z; Regnier C
AN 1999-04021 BIOTECHDS
PI WO 9901541 14 Jan 1999

L37 ANSWER 3 OF 35 WPIDS (C) 2002 THOMSON DERWENT
TI Screening for agents which modulate the interaction of IKK-beta polypeptides and their binding targets.
PI US 5916760 A 19990629 (199932)* 14p C12P001-48
IN GOEDDEL, D V; WORONICZ, J

L37 ANSWER 4 OF 35 MEDLINE DUPLICATE 2
TI Differential effects of lipopolysaccharide and tumor necrosis factor on monocytic IkappaB kinase signalsome activation and IkappaB proteolysis.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 Aug 27) 274 (35) 24625-32.
Journal code: 2985121R. ISSN: 0021-9258.
AU Fischer C; Page S; Weber M; Eisele T; Neumeier D; Brand K
AN 1999386936 MEDLINE

L37 ANSWER 5 OF 35 MEDLINE DUPLICATE 3
TI IKKgamma serves as a docking subunit of the IkappaB kinase (**IKK**) and mediates interaction of **IKK** with the **human** T-cell leukemia virus Tax protein.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 Aug 13) 274 (33) 22911-4.
Journal code: 2985121R. ISSN: 0021-9258.
AU Harhaj E W; Sun S C
AN 1999367408 MEDLINE

L37 ANSWER 6 OF 35 HCAPLUS COPYRIGHT 2002 ACS
TI IKK.gamma. mediates the interaction of cellular I.kappa.B kinases with the Tax transforming protein of human T cell leukemia virus type 1
SO Journal of Biological Chemistry (1999), 274(22), 15297-15300
CODEN: JBCHA3; ISSN: 0021-9258
AU Chu, Zhi-Liang; Shin, Young-Ah; Yang, Jin-Ming; DiDonato, Joseph A.; Ballard, Dean W.
AN 1999:349294 HCAPLUS
DN 131:141960

L37 ANSWER 7 OF 35 MEDLINE DUPLICATE 4
TI Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates IkappaB kinases (IKK-alpha/beta) and IKK-beta is a developmentally regulated protein kinase.
SO ONCOGENE, (1999 Sep 30) 18 (40) 5514-24.
Journal code: 8711562. ISSN: 0950-9232.
AU Hu M C; Wang Y p; Qiu W R; Mikhail A; Meyer C F; Tan T H
AN 1999455228 MEDLINE

L37 ANSWER 8 OF 35 MEDLINE DUPLICATE 5
TI Primary human CD4+ T cells contain heterogeneous I kappa B kinase complexes: role in activation of the IL-2 promoter.
SO JOURNAL OF IMMUNOLOGY, (1999 Nov 15) 163 (10) 5444-52.
Journal code: 2985117R. ISSN: 0022-1767.
AU Khoshnan A; Kempiak S J; Bennett B L; Bae D; Xu W; Manning A M; June C H; Nel A E
AN 2000021841 MEDLINE

L37 ANSWER 9 OF 35 SCISEARCH COPYRIGHT 2002 ISI (R)
TI Differential expression of I kappa B alpha, **IKK** alpha, **IKK** beta and **IKK** gamma in various **human** cell lines and different tissues.
SO MOLECULAR BIOLOGY OF THE CELL, (NOV 1999) Vol. 10, Supp. [S], pp. 2456-2456.
Publisher: AMER SOC CELL BIOLOGY, PUBL OFFICE, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814.

ISSN: 1059-1524.
AU Wu C (Reprint); Dias P; Stampfl J; Tevelde E; Oania R; Tan W; Stampfl C; Lopez C; Kumar S; Singh S
AN 1999:981904 SCISEARCH

L37 ANSWER 10 OF 35 MEDLINE DUPLICATE 6
TI IkappaB kinase complex is an intracellular target for endotoxic lipopolysaccharide in human monocytic cells.
SO BLOOD, (1999 Sep 1) 94 (5) 1711-6.
Journal code: 7603509. ISSN: 0006-4971.
AU Hawiger J; Veach R A; Liu X Y; Timmons S; Ballard D W
AN 1999409043 MEDLINE

L37 ANSWER 11 OF 35 HCAPLUS COPYRIGHT 2002 ACS
TI IkB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex
SO Molecular and Cellular Biology (1999), 19(2), 1526-1538
CODEN: MCEBD4; ISSN: 0270-7306
AU Mercurio, Frank; Murray, Brion W.; Shevchenko, Andrej; Bennett, Brydon L.; Young, David B.; Li, Jian Wu; Pascual, Gabriel; Motiwala, Aparna; Zhu, Hengyi; Mann, Matthias; Manning, Anthony M.
AN 1999:106311 HCAPLUS
DN 130:263791

L37 ANSWER 12 OF 35 HCAPLUS COPYRIGHT 2002 ACS
TI IKK-i, a novel lipopolysaccharide-inducible kinase that is related to I.kappa.B kinases
SO International Immunology (1999), 11(8), 1357-1362
CODEN: INIMEN; ISSN: 0953-8178
AU Shimada, Takahiro; Kawai, Taro; Takeda, Kiyoshi; Matsumoto, Makoto; Inoue, Jun-Ichiro; Tatsumi, Yoichi; Kanamaru, Akihisa; Akira, Shizuo
AN 1999:511669 HCAPLUS
DN 131:297982

L37 ANSWER 13 OF 35 MEDLINE DUPLICATE 7
TI Activation and regulation of the IkappaB kinase in human B cells by CD40 signaling.
SO EUROPEAN JOURNAL OF IMMUNOLOGY, (1999 Apr) 29 (4) 1353-62.
Journal code: 1273201. ISSN: 0014-2980.
AU Kosaka Y; Calderhead D M; Manning E M; Hambor J E; Black A; Geleziunas R; Marcu K B; Noelle R J
AN 1999244109 MEDLINE

L37 ANSWER 14 OF 35 MEDLINE DUPLICATE 8
TI IKK1-deficient mice exhibit abnormal development of skin and skeleton.
SO GENES AND DEVELOPMENT, (1999 May 15) 13 (10) 1322-8.
Journal code: 8711660. ISSN: 0890-9369.
AU Li Q; Lu Q; Hwang J Y; Buscher D; Lee K F; Izpisua-Belmonte J C; Verma I M
AN 1999276562 MEDLINE

L37 ANSWER 15 OF 35 MEDLINE DUPLICATE 9
TI Carbachol activates IkappaB kinase in isolated canine gastric parietal cells.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1999 Aug 11) 261 (3) 877-84.
Journal code: 0372516. ISSN: 0006-291X.
AU Todisco A; Ramamoorthy S; Pausawasdi N; Tacey K
AN 1999373172 MEDLINE

L37 ANSWER 16 OF 35 HCAPLUS COPYRIGHT 2002 ACS
TI Suppression of TNF.alpha.-mediated NF.kappa.B activity by myricetin and other flavonoids through down-regulating the activity of IKK in ECV304 cells
SO Journal of Cellular Biochemistry (1999), 74(4), 606-615

CODEN: JCEBD5; ISSN: 0730-2312
AU Tsai, Shu-Huei; Liang, Yu-Chih; Lin-Shiau, Shoei-Yn; Lin, Jen-Kun
AN 1999:513970 HCAPLUS
DN 131:266780

L37 ANSWER 17 OF 35 MEDLINE DUPLICATE 10
TI NF-kappa B regulation by I kappa B kinase in primary fibroblast-like synoviocytes.
SO JOURNAL OF IMMUNOLOGY, (1999 Jul 1) 163 (1) 427-33.
Journal code: 2985117R. ISSN: 0022-1767.
AU Aupperle K R; Bennett B L; Boyle D L; Tak P P; Manning A M; Firestein G S
AN 1999316333 MEDLINE

L37 ANSWER 18 OF 35 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
TI Differential expression of IkappaBalpha, **IKKalpha**, **IKKbeta**, and **IKKgamma** in various **human** cell lines and different tissues.
SO Molecular Biology of the Cell, (Nov., 1999) Vol. 10, No. SUPPL., pp. 424a.
Meeting Info.: 39th Annual Meeting of the American Society for Cell Biology Washington, D.C., USA December 11-15, 1999 The American Society for Cell Biology
. ISSN: 1059-1524.
AU Wu, Chun; Dias, Peter (1); Stampfl, Jason (1); Tevelde, Eric (1); Oania, Robert (1); Tan, Winny (1); Stampfl, Chris (1); Lopez, Carlos (1); Kumar, Shant; Singh, Sujay (1)
AN 2000:83411 BIOSIS

L37 ANSWER 19 OF 35 MEDLINE DUPLICATE 11
TI Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice.
SO IMMUNITY, (1999 Apr) 10 (4) 421-9.
Journal code: 9432918. ISSN: 1074-7613.
AU Tanaka M; Fuentes M E; Yamaguchi K; Durnin M H; Dalrymple S A; Hardy K L; Goeddel D V
AN 1999244191 MEDLINE

L37 ANSWER 20 OF 35 MEDLINE DUPLICATE 12
TI Identification of the gene variations in **human IKKA**.
SO IMMUNOGENETICS, (1999 Dec) 50 (5-6) 363-5.
Journal code: 0420404. ISSN: 0093-7711.
AU Hagiwara K; Tsuchiya N; Takazoe M; Yamamoto K; Tokunaga K
AN 2000093999 MEDLINE

L37 ANSWER 21 OF 35 MEDLINE DUPLICATE 13
TI Severe liver degeneration in mice lacking the IkappaB kinase 2 gene.
SO SCIENCE, (1999 Apr 9) 284 (5412) 321-5.
Journal code: 0404511. ISSN: 0036-8075.
AU Li Q; Van Antwerp D; Mercurio F; Lee K F; Verma I M
AN 1999212144 MEDLINE

L37 ANSWER 22 OF 35 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
TI Limb and skin abnormalities in mice lacking IKKalpha.
SO Science (Washington D C), (April 9, 1999) Vol. 284, No. 5412, pp. 313-316.
ISSN: 0036-8075.
AU Takeda, Kiyoshi; Takeuchi, Osamu; Tsujimura, Tohru; Itami, Satoshi; Adachi, Osamu; Kawai, Taro; Sanjo, Hideki; Yoshikawa, Kunihiko; Terada, Nobuyuki; Akira, Shizuo (1)
AN 1999:227301 BIOSIS

L37 ANSWER 23 OF 35 SCISEARCH COPYRIGHT 2002 ISI (R) DUPLICATE 14
TI Nuclear factor kappa B in liver disease
SO GUT, (AUG 1999) Vol. 45, No. 2, pp. 170-171.
Publisher: BRITISH MED JOURNAL PUBL GROUP, BRITISH MED ASSOC HOUSE, TAVISTOCK SQUARE, LONDON WC1H 9JR, ENGLAND.

ISSN: 0017-5749.
AU Gallagher N J (Reprint); Young L S
AN 1999:589545 SCISEARCH

L37 ANSWER 24 OF 35 BIOTECHDS COPYRIGHT 2002 THOMSON DERWENT AND ISI
TI Nucleic acid encoding I-kappa-B-kinase subunits and antibodies; the subunits phosphorylate the inhibitor of NF-kappa-B, for studying the inflammatory response and signal response and signal transduction pathway
AU Karin M; DiDonato J A; Rothwarf D M; Hayakawa M; Zandi E
AN 1998-10437 BIOTECHDS
PI WO 9837228 27 Aug 1998

L37 ANSWER 25 OF 35 BIOTECHDS COPYRIGHT 2002 THOMSON DERWENT AND ISI
TI DNA encoding IKK-beta polypeptides; recombinant I-kappa-B kinase preparation, DNA probe and DNA primer, used for disease diagnosis therapy or gene therapy
AU Goeddel D V; Woronicz J
AN 1999-03288 BIOTECHDS
PI US 5851812 22 Dec 1998

L37 ANSWER 26 OF 35 BIOTECHDS COPYRIGHT 2002 THOMSON DERWENT AND ISI
TI New isolated stimulus-inducible IkappaB kinase signalsome; recombinant protein production and nucleic acid for use in therapy and gene therapy
AU Mercurio F; Zhu H; Barbosa M; Li G; Murray B W
AN 1998-05570 BIOTECHDS
PI WO 9808955 5 Mar 1998

L37 ANSWER 27 OF 35 MEDLINE DUPLICATE 15
TI Role of **IKK1** and **IKK2** in lipopolysaccharide signaling in **human** monocytic cells.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Nov 13) 273 (46) 30410-4. Journal code: 2985121R. ISSN: 0021-9258.
AU O'Connell M A; Bennett B L; Mercurio F; Manning A M; Mackman N
AN 1999023967 MEDLINE

L37 ANSWER 28 OF 35 MEDLINE DUPLICATE 16
TI NF-kappaB-inducing kinase and IkappaB kinase participate in human T-cell leukemia virus I Tax-mediated NF-kappaB activation.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Aug 14) 273 (33) 21132-6. Journal code: 2985121R. ISSN: 0021-9258.
AU Uhlik M; Good L; Xiao G; Harhaj E W; Zandi E; Karin M; Sun S C
AN 1998361989 MEDLINE

L37 ANSWER 29 OF 35 MEDLINE DUPLICATE 17
TI Human T-cell leukemia virus type 1 Tax induction of NF-kappaB involves activation of the IkappaB kinase alpha (IKKalpha) and IKKbeta cellular kinases.
SO MOLECULAR AND CELLULAR BIOLOGY, (1998 Sep) 18 (9) 5157-65. Journal code: 8109087. ISSN: 0270-7306.
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=>
=> d ab 5,6,12,30

L37 ANSWER 5 OF 35 MEDLINE DUPLICATE 3
AB The tax gene product of human T-cell leukemia virus type I induces activation of transcription factor NF-kappaB, which contributes to deregulated expression of various cellular genes. Tax expression triggers persistent phosphorylation and degradation of the NF-kappaB inhibitory proteins IkappaBalpha and IkappaBbeta, resulting in constitutive nuclear expression of NF-kappaB. Recent studies demonstrate that Tax activates the IkappaB kinase (IKK), although the underlying mechanism remains unclear. In this report, we show that Tax physically interacts with a regulatory component of the IKK complex, the NF-kappaB essential modulator or IKKgamma (NEMO/IKKgamma). This molecular interaction appears to be important for recruiting Tax to the IKK catalytic subunits, IKKalpha and

IKKbeta. Expression of NEMO/IKKgamma greatly promotes binding of Tax to IKKalpha and IKKbeta and stimulates Tax-mediated IKK activation. Interestingly, a mutant form of Tax defective in IKK activation exhibited a markedly diminished level of NEMO/IKKgamma association. These findings suggest that the physical interaction of Tax with NEMO/IKKgamma may play an important role in Tax-mediated IKK activation.

L37 ANSWER 6 OF 35 HCAPLUS COPYRIGHT 2002 ACS
AB The Tax oncoprotein of human T cell leukemia virus type 1 constitutively activates transcription factor NF-.kappa.B by a mechanism involving Tax-induced phosphorylation of I.kappa.B.alpha., a labile cytoplasmic inhibitor of NF-.kappa.B. To trigger this signaling cascade, Tax assocs. stably with and persistently activates a cellular I.kappa.B kinase (IKK) contg. both catalytic (IKK.alpha. and IKK.beta.) and noncatalytic (IKK.gamma.) subunits. We now demonstrate that IKK.gamma. enables Tax to dock with the IKK.beta. catalytic subunit, resulting in chronic I.kappa.B kinase activation. Mutations in either IKK.gamma. or Tax that prevent formation of these higher order Tax.cntdot.IKK complexes also interfere with the ability of Tax to induce IKK.beta. catalytic function in vivo. Deletion mapping studies indicate that amino acids 1-100 of IKK.gamma. are required for this Tax targeting function. Together, these findings identify IKK.gamma. as an adaptor protein that directs the stable formation of pathol. Tax.cntdot.IKK complexes in virally infected T cells.

L37 ANSWER 12 OF 35 HCAPLUS COPYRIGHT 2002 ACS
AB Using the suppression subtractive hybridization technique, the authors isolated a novel kinase, IKK-i, whose message is drastically induced by lipopolysaccharide (LPS) in the mouse macrophage cell line RAW264.7. The predicted protein contains the kinase domain in its N-terminus, which shares 30% identity to that of IKK-.alpha. or IKK-.beta.. The C-terminal portion contains a leucine zipper and a potential helix-loop-helix domain, as in the case of IKK-.alpha. and IKK-.beta.. IKK-i is expressed mainly in immune cells, and is induced in response to proinflammatory cytokines such as tumor necrosis factor-.alpha., IL-1 and IL-6, in addn. to LPS. Overexpression of wild-type IKK-i phosphorylated serine residues Ser32 and Ser36 of I.kappa.B-.alpha. (preferentially Ser36), and significantly stimulated NF-.kappa.B activation. These results suggest that IKK-i is an inducible I.kappa.B kinase which may play a special role in the immune response.

L37 ANSWER 30 OF 35 MEDLINE DUPLICATE 18
AB Pro-inflammatory cytokines activate the transcription factor NF-kappaB by stimulating the activity of a protein kinase that phosphorylates IkappaB, an inhibitor of NF-kappaB, at sites that trigger its ubiquitination and degradation. This results in the nuclear translocation of freed NF-kappaB dimers and the activation of transcription of target genes. Many of these target genes code for immunoregulatory proteins. A large, cytokine-responsive IkappaB kinase (IKK) complex has been purified and the genes encoding two of its subunits have been cloned. These subunits, IKK-alpha and IKK-beta, are protein kinases whose function is needed for NF-kappaB activation by pro-inflammatory stimuli. Here, by using a monoclonal antibody against IKK-alpha, we purify the **IKK** complex to homogeneity from **human** cell lines. We find that **IKK** is composed of similar amounts of IKK-alpha, IKK-beta and two other polypeptides, for which we obtained partial sequences. These polypeptides are differentially processed forms of a third subunit, IKK-gamma. Molecular cloning and sequencing indicate that IKK-gamma is composed of several potential coiled-coil motifs. IKK-gamma interacts preferentially with IKK-beta and is required for the activation of the IKK complex. An IKK-gamma carboxy-terminal truncation mutant that still binds IKK-beta blocks the activation of IKK and NF-kappaB.

=> log y

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